THE UNIVERSITY OF MANITOBA

THE ENZYMIC RELEASE OF FATTY ACIDS IN *VICIA FABA MINOR*

BY

DAVID LAWRENCE SHAMBROCK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

WINNIPEG, MANITOBA

SEPTEMBER 1979

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ABSTRACT

The purpose of this study was to determine if the deacylation of a variety of natural and synthetic lipid substrates could be demonstrated in preparations from fababean (Vicia faba L. var. minor). These enzymic activities are described with the general name of "Lipolytic Acyl Hydrolase". Lipolytic acyl hydrolase is believed to be one of the enzymes active in a degradative pathway responsible for the development of rancidity and off-flavor in processed fababean.

Initial attempts at purifying the enzyme (acetone fractionation) were unsuccessful, decreasing the specific activity. A crude extract was used for the kinetic studies. A wide selection of p-nitrophenyl fatty acid ester and phospholipid substrates were assayed with the enzyme. For assays with p-nitrophenyl esters, activity was measured by following the release of p-nitrophenol by means of a spectrophotometer. The release of fatty acids from the phospholipid substrates was measured by determining the corresponding decrease in acyl ester content of the phospholipids.

The course of the hydrolytic reaction was linear with respect to enzyme concentration. With the p-nitrophenyl esters, the enzyme demonstrated greater activity with the shorter chain derivatives. Km values were determined to be 1.43 mM (p-nitrophenyl acetate), 0.63 mM (p-nitrophenyl palmitate) and 1.82 mM (p-nitrophenyl stearate). In terms of phospholipase activity Km values of 0.18 mM for the hydrolysis of phosphatidylcholine and 0.73 mM for 1,2 dilinolein hydrolysis were obtained. Difficulties were encountered in preparing certain of the emulsified substrates.

Commercial preparations of hog pancreatic lipase were assayed

with the standard phospholipase assay procedure. The low activity observed tends to indicate that fababean phospholipase activity may be distinct from a lipase fraction in the crude extract.

Tests were run with glycerophosphate to test the specificity of the acyl ester group determination. Negative results were obtained (no color development) indicating that a phospholipase A- or B-type activity may be present in fababean.

The optimum pH for phospholipase activity was 5.6, with a possible secondary optimum of 7.5 indicated. With the p-nitrophenyl ester substrates, optimal activity was pH 8.5.

The optimum temperature for fababean lipolytic acyl hydrolase activity was determined to be 37°C. In solution the enzyme was completely inactivated by exposure to 75°C for two minutes, indicating a comparatively heat-labile enzyme.

The non-ionic detergent Triton X-100 was used to emulsify the lipid substrates. The optimum concentration of 6 mg. Triton/ml. was determined for hydrolysis of p-nitrophenyl esters. For assays with phospholipid substrates the optimum concentration of Triton was found to be 4 mg./ml.

The fababean enzyme reacted quite differently to an activator (calcium chloride) and an inhibitor (mercuric chloride) depending on the substrate assayed. Fababean hydrolysis of p-nitrophenyl palmitate was stimulated by the calcium ion up to a 3 mM concentration, and completely inhibited by a 5 mM concentration of mercuric chloride. Phosphatidylcholine degradation was not affected by additional calcium ion (up to 3 mM) and only 6% of activity was inhibited by 5 mM mercuric chloride.

ACKNOWLEDGEMENT

I would like to take this opportunity to express my gratitude to my advisor, Dr. H. Michael Henderson for his constant encouragement and guidance throughout the course of this study, and for his critical and helpful review of this manuscript.

Thanks are also due to Dr. Ernst Hoehn, Department of Food Science, University of Manitoba, for his able and willing advice offered in many theoretical and practical aspects of this work.

I would like to thank Dr. R.A. Gallop and Dr. E.D. Murray for giving me the opportunity to carry out this research.

For financial assistance in this study, I would like to thank the University of Manitoba Graduate Fellowship Committee and The Natural Sciences and Engineering Research Council Canada.

The author would like to thank Mrs. Connie Ashdown for typing this manuscript.

Final thanks are offered to Ms. Lena Sky for all the patience and understanding that could fit in two years.

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1. INTRODUCTION

The small fababean, or horse bean, Vicia faba L. var. minor, is becoming an increasingly important special crop for the Prairie region of Canada (Presber, 1972; Manitoba Department of Agriculture Newsletter, Dec. 22, 1978). Although the fababean is indigenous to Western Europe and the Mediterranean basin, its introduction to northern growing conditions appears favorable from the results of multidisciplinary research undertaken in Manitoba and Saskatchewan. In studies with several cultivars of fababeans, it was found that the mean test yield of the fababeans was higher than that of the wheat cultivar Glenlea (Evans, et al., 1972). Bhatty (1974) found that twelve cultivars of fababean varied in protein content from 26 to 35%. The low fat content (1.0 - 1.5% of dry matter) of the fababean permits dry milling which, with air classification, can offer a high-protein concentrate (McDonald, 1974). While the nutritional potential of the fababean appears attractive, its use in human foods requires clarification of possible toxic fractions (Collier, 1976), and of its palatability. Patel et al., (1977) reported beany and bitter flavor characteristics in fababean flour and protein isolates. The development of rancid odors and flavors in defatted soy products has been associated with the enzyme lipoxygenase (Mustakas et al., 1967). Eskin and Henderson (1974a;b) confirmed the presence in fababeans of an active lipoxygenase, which appeared to exhibit a high degree of specificity towards polyunsaturated free fatty acids. In intact fababeans, fatty acids are present predominantly esterified to the alcohol (glycerol) moiety of either triglycerides or phospholipids. Hinchcliffe et al. (1977) reported the increase of free fatty acids

during the accelerated storage of fababeans as either a concentrate or flour, indicating the presence of esterases, which would hydrolyse the ester linkages releasing the fatty acids. Since the action of a lipase which deacylated triglycerides was previously studied in the fababean (Dundas et al., 1978) it was decided to investigate the mechanism of release of free fatty acid from the phospholipid fraction. Phospholipids comprise the major lipid fraction in the fababean (66%), while triacylglycerols (triglycerides) account for 30% of fababean lipids (Hinchcliffe et al., 1977). Many different enzymes can deacylate phospholipids, however they are all included in the general name "Lipolytic Acyl Hydrolase". The presence of phospholipase D, which releases choline from phosphatidylcholine has recently been confirmed in the fababean (Atwal et al., 1979).

Studies with lipolytic acyl hydrolases from plant sources have been concerned primarily with lipid degradation upon disruption of plant tissues. The enzymes appear to be in an inactive state, or at least separated from the lipid substrates, prior to cell disruption. In the literature, reports on acyl hydrolases are confusing, the same enzyme being given different names by different authors. The main problem area is the use of a limited number of substrates in a vast number of assay conditions and procedures.

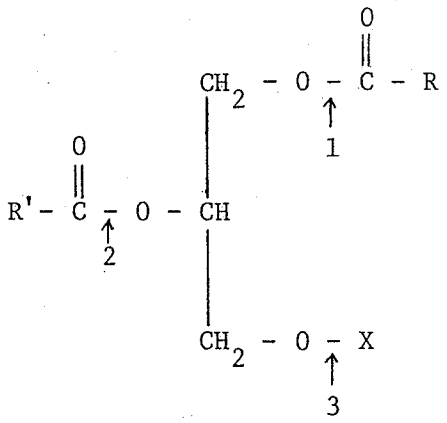
The object of this study was the detection of acyl hydrolase activity in fababean, and the characterization of the enzyme. The effect of certain conditions (pH, temperature, activators and inhibitors) on lipolytic acyl hydrolase activity was investigated.

2. REVIEW OF LITERATURE

The enzyme lipolytic acyl hydrolase belongs to the widely-distributed group of enzymes known as esterases, which are involved in the splitting of ester linkages by the addition of water. The name 'lipolytic acyl hydrolase' is a trivial one, and has been used in the literature to describe enzyme activities that differ markedly from each other. Plant enzymes that catalyze the deacylation of lipids have been variously described as lipases, phospholipases, galactolipases and esterases. Unequivocal characterization of specific acyl hydrolases, with the exception of seed lipases (glycerol ester hydrolase, EC 3.1.1.3), is rare in literature on plant enzymes (Galliard and Dennis, 1974a). The diversity of data is due partly to the use of a limited range of substrates in a wide choice of assay conditions and methods used to determine lipolytic activity. It has been demonstrated that a given lipolytic enzyme will show marked differences in properties and specificity under different assay conditions (e.g., pH, presence of detergents or metal ions) and with different physiological conditions of the plant (Galliard, 1968; 1971). The diversity of data is also due in part to the use of crude enzyme preparations, with the further complicating possibility of isoenzymes which have differing substrate specificities and optimum reaction conditions. Galliard (1971a), using a enzyme preparation that was purified 5.1-fold versus the crude enzyme extract from potato, reported that this preparation catalyzed the deacylation of mono- and di-acyl phospholipids, galactosyl diglycerides, mono- and di-glycerides, as well as methyl and p-nitrophenyl esters. This preparation was later shown by gel and free-flow electrophoresis

to exhibit multiple forms (isoenzymes) of lipolytic acyl hydrolase and esterase enzymes (Galliard and Dennis, 1974b).

Regardless of the impurity of many of the enzyme preparations, it is possible to classify the types of activity ascribed to 'lipolytic acyl hydrolases' within the classification recommended by the Enzyme Commission (Florkin and Stotz, 1965). All lipolytic enzymes are hydrolases and therefore belong to class 3 within this system. Lipolytic acyl hydrolases are ester hydrolases, enzyme group 3.1. No bonds other than carboxyl ester bonds have ever been found to be hydrolysed and therefore lipolytic acyl hydrolases are further subgrouped as carboxylic-ester hydrolases, enzyme group 3.1.1. At this point the classification branches depending on the substrate, and the positional specificity of the particular enzyme. Polar glycerolipids with the following formula:



where R and R' represent fatty acyl residues and X represents a phosphate ester in phospholipids, or a glycosidic moiety in glycolipids, may be deacylated by the following enzymes. In the case of phospholipids, phosphatide acyl-hydrolase, (EC 3.1.1.4) hydrolyses the acyl-ester linkage at position 2, releasing fatty acid R' COOH and resulting in the production of a lysophospholipid. The recommended trivial name for this enzyme is Phospholipase A. Lysolecithin acyl-

hydrolase (EC 3.1.1.5) hydrolyses the acyl-ester linkage at position 1 releasing fatty acid R COOH and producing a glycerolphospholipid. The recommended trivial name for this enzyme is Lysophospholipase. The term phospholipase "B" was originally applied to an enzyme that removed both acyl groups from a diacyl phospholipid, although available evidence (Ansell and Hawthorne, 1964) indicated that this activity could be ascribed to a mixture of two enzymes (phospholipases A_1, EC 3.1.1.32 and phospholipase A_2, EC 3.1.1.4) each specific for one of the two acyl ester bonds. Subsequently, phospholipase B has been defined as an alternative name for lysophospholipase (EC 3.1.1.5) (Ansell and Hawthorne, 1964; Florkin and Stotz, 1973).

Referring again to the above general formula, in the case of glycolipids (X represents a glycosidic moiety), the enzyme deacylating this lipid is classified as Galactosylglyceride acyl-hydrolase (EC 3.1.1.26) with the trivial name galactolipase. There appears to be no positional specificity, the enzyme deacylating at position 1 and 2, although there is evidence that this enzyme is actually identical with a mixture of phospholipases A_1 and A_2 (Galliard and Dennis, 1974a; Hirayama et al., 1975; Hasson and Laties, 1976a). If X represented a third fatty acyl residue, the enzyme deacylating at positions 1, 2 or 3 would be glycerol-ester hydrolase, (EC 3.1.1.3) commonly referred to as lipase.

2.1 Distribution of Lipolytic Acyl-Hydrolases

For the purpose of clarity this discussion will concern itself with those enzymes reported in the literature as galactolipases, phospholipases A, A_1, A_2 or B, lysophospholipase and lipolytic acyl hydrolases. Although lipolytic acyl hydrolases have been studied from a variety

of animal, microbiological and plant sources, some in great detail, there is still much confusion concerning the characteristics and specificities of these enzymes.

2.1.1 Animal Lipolytic Acyl Hydrolases

Lipolytic enzymes isolated from the tissues or fluids from a variety of animal sources have been studied extensively for many years. Phospholipase activity has been investigated in bovine milk (Scow and Egelrud, 1976; Eisenberg et al., 1978) rat plasma (Eisenberg and Schurr, 1976; Fielding et al., 1977), rat heart (Chajek and Eisenberg, 1978), rat intestinal mucosa (Subbaiah and Ganguly, 1970), rat stomach mucosa (Wassef et al., 1978), rat hepatocytes (Kanoh and Åkesson, 1977), an inflammatory exudate from rabbit (Franson et al., 1978), porcine pancreas (de Haas et al., 1968), sheep erythrocyte membranes (Frei and Zahler, 1979), and various snake venoms (Saito and Hanahan, 1962; Colacicco and Rapport, 1966; Shiloah et al., 1973a).

The enzyme phospholipase A_2 (EC 3.1.1.4) is the well-known phospholipase of snake venom. The enzyme specifically attacks at the 2 position of the glycerol moiety regardless of whether the fatty acid bound there is saturated or unsaturated. Enzymes carrying out this reaction have been discovered in a number of animal tissues (Gallai-Hatchard and Thompson, 1965). Although the degree of activity varies widely, it is likely that the enzyme is almost ubiquitous (Florkin and Stotz, 1970).

Phospholipase A_1 (EC 3.1.1.32) has been separated from phospholipase A_2 in homogenates of rat liver by Waite and Van Deenan (1964). The properties of the two enzymes were shown to be different and distinct. The phospholipase A_1 also appears to be different from

the enzyme pancreatic lipase which has also been shown to attack the 1-acyl ester of phospholipids (Sarda et al., 1964). A phospholipase A_1 activity has also been isolated from brain (Gatt, 1968) and from human plasma (Vogel and Bierman, 1967).

The enzyme lysophospholipase, also termed lysolecithin acyl-hydrolase (EC 3.1.1.5) and sometimes phospholipase B, has been the subject of some confusion. Although a phospholipase B-type of enzyme (deacylating at both 1 and 2 positions of phospholipids) is still considered to be the reaction catalyzed by the Penicillium notatum enzyme (Dawson and Hauser, 1967), such activity in animal tissues has been questioned (Ansell and Hawthorne, 1964). The Enzyme Commission of the International Union of Biochemistry has listed phospholipase B as a synonym for lysolecithin acyl-hydrolase, an enzyme catalyzing the following reaction:

$$\text{Lysolecithin} + H_2O \longrightarrow \text{Glycerylphosphorylcholine} + \text{Fatty Acid.}$$

No specificity is indicated with respect to the position which can be attacked (i.e., the C-1 or C-2 position of the glyceryl moiety). However, work by DeJong et al. (1973) has indicated that lysophospholipases may have a much wider range of substrates.

2.1.2 Microbial Lipolytic Acyl Hydrolases

Phospholipases are present in many Gram-negative bacteria located chiefly in the membrane. They are similar to venom and tissue phospholipase A_2. The great affinity of these enzymes for membrane phospholipid reflects their participation in its metabolism (Bernard et al., 1972). The presence of a phospholipase A in Escherichia coli was first reported by Okueyama and Nojima (1969). Since then much work has been done with various strains of this micro-organism to

determine acyl group specificity. Proulx and Fung (1969) found both A_1 and A_2 activity, as did Doi and Nojima (1973). Patriarca et al. (1972) and Scandella and Kornberg (1971) both found phospholipase A_1 activity, while Bernard et al. (1972) found phospholipase A_2 activity. More recently, Nishijima et al. (1977) isolated an enzyme that showed phospholipase A_1 and A_2 activity as well as lysophospholipase activity at both the 1 and 2 positions of the glyceryl moiety.

Phospholipase activity has also been described in other organisms such as Clostridium welchii (Nakamura et al., 1969) and in toxic preparations from Staphylococcus aureus (Doery et al., 1965).

2.1.3 Plant Lipolytic Acyl Hydrolases

Compared with the literature concerning lipolytic enzymes isolated from microbial and animal sources, reports on plant lipolytic acyl hydrolases are substantially fewer. Recent interest in plant deacylating enzymes has resulted largely from the work of Galliard (1970), who reported that homogenation of potato tubers at 0°C results in rapid enzymic hydrolysis of the endogenous phospholipids and galactolipids to produce free fatty acids and fatty acid hydroperoxides. The production of fatty acid hydroperoxides was attributed to the presence of lipoxygenase (EC 1.13.11.12), while the enzymes responsible for the catabolism of phospholipids and galactolipids were named phospholipid- and galactolipid acyl hydrolases, respectively.

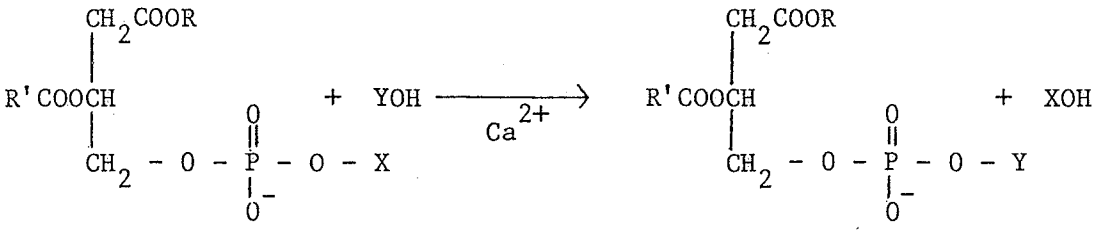
Since this initial report, considerable work has been done with the potato tuber, whose tissues have the highest reported activity for lipolytic acyl hydrolases (Galliard 1971a, 1971b; Galliard and Matthew, 1973; Galliard and Dennis, 1974a; 1974b; Shepard and Pitt, 1974a, 1974b; Hirayama et al., 1975; Hasson and Laties, 1974a; 1976b).

Work has been done with other plant sources including alfalfa and corn roots (Yagi and Benson, 1962), germinating barley (Acker and Bücking, 1956; Acker and Geger, 1969; Rebman and Acker, 1973), cucumber (Galliard et al., 1976), tomato (Galliard et al., 1977), spinach (Hirayama and Oida, 1969; Duden et al., 1977), cereal grains (Nolte et al., 1974), rice bran (Hirayama and Matsuda, 1975) and rice endosperm (Matsuda and Hirayama, 1975).

It is important to note that in no case has an absolute specificity for a given class of lipid been established.

2.1.3.1 Phospholipase D in Plants

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is the only fully-characterized enzyme from plant tissues which attacks polar groups of acyl lipids. A complete review of the available information concerning the enzyme may be found in the reference of Galliard (1973). Phospholipase D catalyses the following general reaction:



where R and R' represent fatty acid residues and YOH may be water or an alcohol acceptor. Thus, the enzyme catalyses both hydrolysis and transphosphatidylation reactions. X represents a wide range of molecules - organic bases, amino acids and alcohols.

Phospholipase D has been shown to catalyse the hydrolysis of most of the common phospholipids with the exception of

phosphoinositides, but the reaction conditions for hydrolysis *in vitro* vary markedly for different substrates (Galliard, 1973). The physical state of the substrate dispersion is critical for enzyme activity and this is dependent on the nature of the polar group, chain length and unsaturation of the fatty acyl moieties, pH, concentration of substrate, and the presence of surface agents (Chen and Barton, 1971; Dawson, 1973). The physiological role of phospholipase D is not known. Although activity is high in certain storage tissues (the stalk of cabbage and celery, the root of carrot, seeds of pea and marrow and the tuber of Jerusalem artichokes), this generalization is not rigid because the enzyme is absent or low in potato tuber and many fruits (Quarles and Dawson, 1969). The enzyme has been demonstrated in the seeds of peanut (Tzur and Shapiro, 1972; Strauss *et al.*, 1976) and recently in the fababean (Atwal *et al.*, 1979).

2.2 Substrates

Lipolytic acyl hydrolases hydrolyse emulsified polar glycerolipids, which are saturated or unsaturated fatty acid esters of glycerolphospholipids and glycolipids. Monoglycerides may be grouped with the polar lipids in relation to hydrolysis with acyl hydrolases. The deacylation of monoglycerides by enzymes distinct from true lipases has been described in seeds of wheat (Stauffer and Glass, 1966), castor bean (Muto and Beevers, 1974) and in potato tuber (Galliard, 1971a). This class of lipolytic enzyme also hydrolyses "artificial" substrates such as methyl and p-nitrophenyl esters of fatty acids.

The reaction of lipolytic enzymes should apply to the rate constants of kinetic equations and it has been demonstrated that the hydrolysis of various lipid groups by potato tuber lipolytic acyl hydrolase obeys the fundamental Michaelis-Menten equation, albeit with marked deviations at higher substrate concentrations (Galliard, 1971a). However, the kinetics of lipolytic reactions are more complicated than at first indicated. Conventional enzyme kinetics have been developed for reactions in aqueous solutions. The enzymic hydrolysis of lipids is unconventional in the sense that it is a heterogeneous reaction because the enzyme is water-soluble but its substrate is not. Therefore, the enzyme-substrate interaction must take place at the interface of the aggregated substrate and water.

One of the major problems in interfacial kinetics of lipolytic enzymes acting on water-insoluble substrates is the expression of a relevant "substrate concentration". Even when these enzymes act on water soluble short-chain substrates which form micelles in solution, having all their lipid molecules equally exposed to solvent and enzyme, the kinetically determined Vmax and Km values should be considered as apparent (Verger et al., 1973). By increasing the amount of substrate, the number of micelles increases, offering a larger lipid-water interface to the enzyme. More enzyme will be bound to the interface and the velocity of the hydrolytic reaction increases. This reversible adsorption or penetration step of the enzyme into the interface is described in Figure 1 by the equilibrium $E \rightleftharpoons E^*$ characterized by the rate constants k_p and k_d. Once bound to the interface, the enzyme (E*) finds a substrate concentration which, expressed as a number of molecules/cm^2, is constant

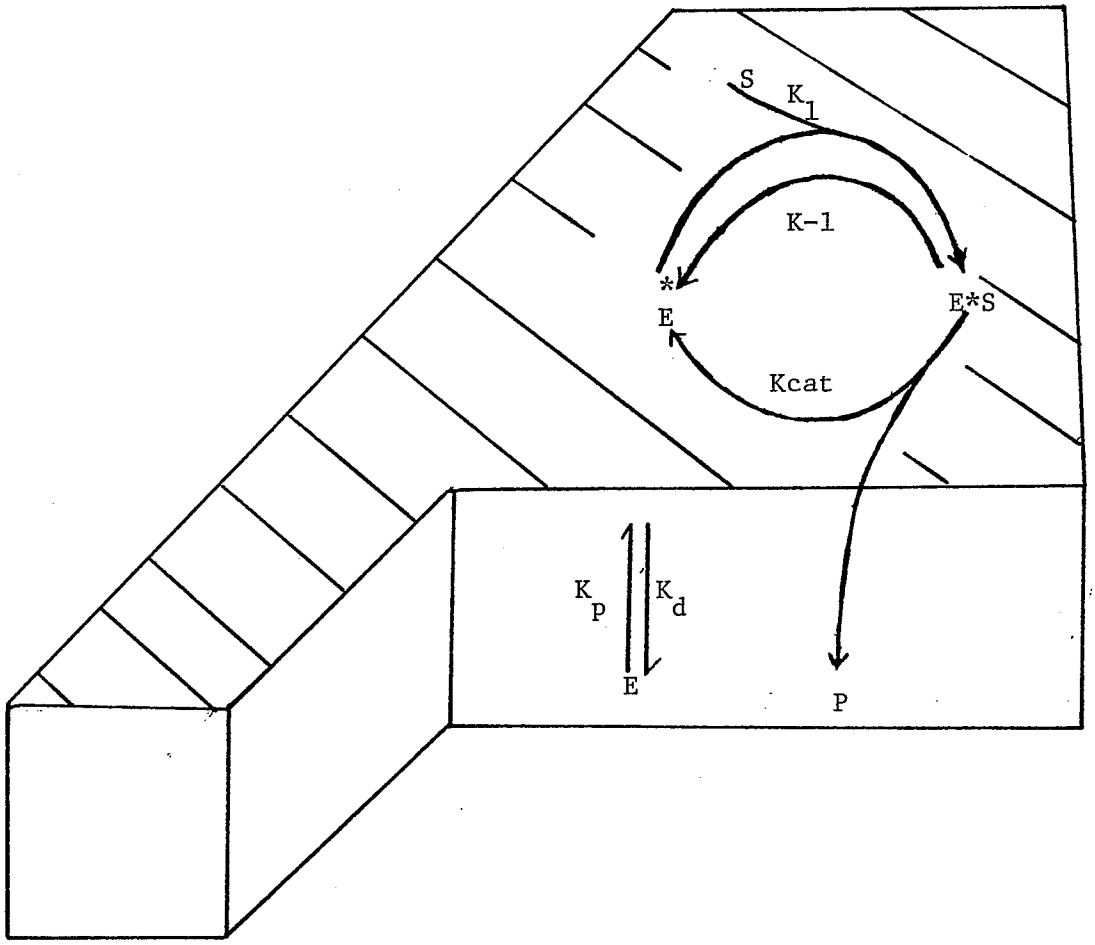


Figure 1. Model for the Action of a Soluble Enzyme at an Interface (Verger et al., 1973).

and cannot easily be varied without changing at the same time the "quality of the interface" and therefore k_p and k_d (Verger et al., 1973).

Therefore, the interfacial K_m value determined by bulk kinetics should be considered as an apparent dissociation constant between enzyme and interface determined not only by k_p and k_d but dependent on k_{-1}, k_1 and k_{cat} as well. For similar reasons the experimentally observed Vmax values should also be considered as apparent. Even if all enzyme is present as E*, i.e., bound to the micellar interface, no real maximal velocity can be derived because in the interface it is not possible to increase the two-dimensional substrate concentration, that is the number of molecules per unit surface (Verger et al., 1973).

2.2.1 The Application of Triton X-100

Phospholipase A_2, as well as other lipolytic enzymes, acts in vivo on substrates that are part of macromolecular aggregates. For in vitro kinetic studies, several forms of the substrate have been employed including monolayers (Verger et al., 1973), alkanol-activated bilayers (Goldhammer et al., 1975), micelles of phospholipids containing short chain fatty acids (deHaas et al., 1971), monomers (Wells, 1972), ether-water complexes (Misiorowski and Wells, 1974) and mixed micelles with surfactants (Dennis, 1973a).

The Triton X-100-phosphatidylcholine mixed micelle system allows a direct kinetic investigation of the interaction of the enzyme and the lipid-water interface, because it provides a system in which the concentration of substrate in the interface can be varied and the activity can be followed by standard kinetic techniques

(Deems et al., 1975).

Triton X-100 is a polydisperse preparation of p-t-octylphenoxy-polyethoxyethanols consisting of oxyethylene chain lengths averaging 9-10 oxyethylene units. However, the general physical properties of this non-ionic detergent are similar to those of the pure homogeneous compound having a chain length of 9 or 10 units (Becher, 1967). Triton X-100 in aqueous solution forms micelles consisting of about 100-160 monomers corresponding to a molecular weight of about 63,000 - 105,000 determined by ultracentrifugation (Dwiggins et al., 1960). Phospholipids such as egg phosphatidylcholine in aqueous dispersion in the concentration range of kinetic studies do not exist as either monomers or micelles (of the detergent type), but rather as large aggregates in the form of bilayers. Detergents are generally thought to solubilize phospholipids by forming mixed phospholipid-detergent micelles, thus drastically altering the physical state of the phosphclipid bilayer (Dennis, 1973b). These changes are maximal when the molar ratio of Triton/phospholipid is in the range of 2:1, and additional Triton X-100 causes no further change in the physical state of the phospholipid. Mixed micelles at a molar ratio of about 10:1 Triton/phospholipid were found to be in the same size range as pure micelles of Triton X-100 (Dennis, 1974).

In the enzymatic studies reported by Dennis (1973b), the activity of phospholipase A_2 depended on the concentration of Triton X-100, with maximal activity requiring a molar ratio of 2:1 Triton/phospholipid. Higher concentrations of Triton X-100 were found to be inhibitory. It was decided that the activity is a complicated function of both phosphatidylcholine and Triton X-100. The

physical state of the phospholipid must be altered by the addition of Triton X-100 in order for the phospholipid substrate to be accessible to the enzyme. Most or all of the phospholipid may be in the altered physical state at a molar ratio of 2:1 Triton/phospholipid. Thus, maximal enzymic activity is observed at a phospholipid concentration and consequently a defined Triton X-100 concentration, which saturates the enzyme. Triton X-100 is widely used as a dispersing agent in studies using purified lipid substrates in aqueous solutions.

2.3 Substrate Specificity

The chemical structure of the substrates usually defines the specificity of an enzyme. In the case of lipolytic acyl hydrolases, however, it is the physical form of the substrate that is used to determine the specificity of each particular enzyme. The substrate specificity of a lipolytic acyl hydrolase is defined by its positional specificity, that is, the ability to hydrolyze the ester bonds at the C1 and/or C2 position of a phospho- or galactolipid; by its preference for longer or shorter saturated or unsaturated fatty acids, and by the sequence of splitting of fatty acids from the glyceryl moieties of the various lipid substrates. Enzymes, in general, vary considerably in their degree of specificity. Some have absolute specificity for a given substrate and will not attack even very closely related molecules, whereas others will attack a whole class of molecules sharing a common denominator of structure but at widely different rates. Lipolytic acyl hydrolases from various plant, animal and microbial sources span this entire range of specificity.

In terms of positional specificity, the lipolytic acyl hydrolases isolated from animal and microbial sources have fared better. Phospholipase A2 is the enzyme of snake venom. Its positional specificity has been well-characterized using highly purified preparations (Shiloah et al., 1973a). Enzymes specific for the C-2 position of phospholipids have been discovered in a number of animal tissues (Gallai-Hatchard and Thompson, 1965), although other properties of the enzymes differ. Doi and Nojima (1973) give a review of the types of enzyme activities, including A1 and A2 specificities, that have been found in various strains of Escherichia coli. Work with animal lysophospholipases have yielded some confusing results. van den Bosch et al. (1973) prepared a highly-purified enzyme from beef pancreas that was specific for the C-1 positon of 1-[1-^{14}C] Palmitoyl-sn-glycero-3-phosphorylcholine. They later found that this enzyme also hydrolysed triacetin, tributyrin, p-nitrophenyl-acetate and short-chain phosphatidylcholines (deJong et al., 1973).

The work done with plant lipolytic acyl hydrolases is very unclear, due mainly to the use of crude enzyme preparations. Galliard (1971a) used partially-purified preparation from potatoes and found hydrolytic activity towards a wide range of substrates, thus showing little or no positional specificity. Later work (Galliard and Dennis, 1974a; Shepard and Pitt, 1976b) resulted in further purification, but gave no further information on specificity other than phospholipase A or B-type activity. In fact, enzyme preparations that were considered pure could not be shown to have an absolute positional specificity (Hirayama et al., 1975; Hasson and Laties, 1976a).

Relatively less information is available concerning the enzyme preferences for chain length and degree of saturation of the fatty acids. This seems unusual considering the wide availability of natural and synthetic substrates that vary considerably in chain length and degree of unsaturation. It should also be considered that natural lecithins show a very marked tendency to contain saturated fatty acids at the C-1 postion and unsaturated fatty acids at the C-2 position (Hanahan and Brockerhoff, 1965). Some information was supplied by Kanok and Åkesson (1977) who used phospholipases from isolated rat hepatocytes to degrade a labelled mixture of different phosphatidylcholines. After 2 hours incubation, about 40% of dilinoleoyl-, 70% of dipalmitoyl- and 30% of dimyristoylglycerophosphocholine were degraded. From the distribution of ^{14}C-labelled fatty acids it was concluded that the degradation of dilinoleoylglycerophosphocholine and that of phosphatidylethanolamine was by the action of phospholipase A1, while the degradation of dipalmitoylglycerophosphocholine proceeded through the action of phospholipase A2. Dimyristoylglycerophosphocholine was probably cleaved by the combined action of both phospholipases A1 and A2. Galliard (1971a) noted that there was a small increase in activity with increased unsaturation and a decrease of activity with higher-chain-length acids (methyl esters of fatty acids as substrates). With p-nitrophenyl esters of fatty acids, he noted no definite trends, although p-nitrophenyl palmitate and octanoate were hydrolyzed most rapidly; p-nitrophenyl acetate was a relatively poor substrate. Hasson and Laties (1976a) found that activity with their potato phospholipase increased with fatty acid chain length. In the

case of a mono- and digalactosyl diglyceride acyl hydrolase isolated from runner bean leaves, the enzyme was found to be highly specific for unsaturated mono- and digalactosyl diglycerides, their saturated counterparts being completely resistant to hydrolysis (Sastry and Kates, 1964). Lack of accessibility of substrate for the enzyme might be a limiting factor due to the fact that the saturated substrates do not form micellar dispersions whereas the unsaturated substrates are readily dispersible.

There is also a lack of conclusive information concerning the sequence of splitting of fatty acids from the glyceryl moiety of phospho- and galactolipids. Shiloah et al. (1973b) found a pronounced preference for lecithin versus lysolecithin, indicating that the C-2 position might be the preferred position for attack. Hirayama et al. (1975) reported conflicting data in this regard. Monogalactosyldiacylglycerol was hydrolysed preferentially over monogalactosylmonoacylglycerol, again indicating the C-2 position as the primary point of attack. However, he also reported that lysophosphatidylcholine was attacked more readily than phosphatidylcholine, indicating that the C-1 position is the primary point of attack. At best, the interpretation of these results is inconclusive and indicates the need for more work.

2.3.1 Wax Ester formation by Lipolytic Acyl Hydrolases

Galliard (1971a) reported that his enzyme preparation would also catalyze the synthesis of wax esters in the presence of a long-chain acid and a long-chain alcohol by the reverse reaction of an acyl-hydrolase. A similar wax synthesis reaction is catalysed by acetone powder preparations of broccoli leaves (Kolattukudy, 1970)

and mammalian liver (Friedberg and Greene, 1967).

The specific activity of the potato lipolytic acyl hydrolase acting hydrolytically on natural lipid substrates (phospholipids, galactolipids, etc.) is a factor of 10^4 higher than its specific activity in wax ester formation (Dennis and Galliard, 1974). This tends to indicate that the equilibrium favors the reverse hydrolysis for wax ester.

2.3.2 Acyl Transferase Activities of Lipolytic Acyl Hydrolases

Galliard (1970) noted that when methanol was present in the incubation mixtures containing potato lipolytic acyl hydrolase, fatty acyl methyl esters were formed in addition to free fatty acids. This indicated a transferase activity by which fatty acids were transferred from the substrate to form an ester with the alcohol present in the mixture. Further study (Galliard and Dennis, 1974a) showed that under appropriate conditions the acyl transferase activity of the enzyme can predominate over the hydrolytic activity. At 10, 20 and 30% methanol concentrations, the affinity of the enzyme for methanol as acyl acceptor is approximately 10-fold over that for water. Thus it was suggested that because of the affinity for an alcohol as acyl acceptor, the enzyme merits a definition of an acyl transferase versus an acyl hydrolase. Fatty acid methyl esters were also produced when a phospholipase A from Escherichia coli was assayed in the presence of methanol (Doi et al., 1972).

2.4 Detection and Assay

The discussion above indicates that the substrates for lipolytic acyl hydrolases must be introduced to the assay medium in an emulsified

form. In principle, a lipolytic reaction can be followed either through disappearance of the substrate, or by the rate of production of the resulting fatty acids or alcohol. The methods used for the quantitative analysis of acyl hydrolase activity can be divided into two groups: (1) assays of the liberated alcohols and (2) assays of the liberated fatty acids.

2.4.1 Assay of the Liberated Alcohols

The methods involving assay of the liberated alcohols are sensitive and fast, being colorimetric or fluorometric in nature. Several reports indicate the use of special substrates that give highly colored end-products after hydrolysis of the acyl ester linkage. Continuous measurement of the p-nitrophenol released by hydrolysis of p-nitrophenyl esters can be obtained with a recording spectrophotometer and the A_{400} related to a standard curve for p-nitrophenol measured at the pH of the incubation (Galliard, 1971a). Alternatively, the p-nitrophenol can be determined from the A_{400} at the end of the incubation period by extracting the p-nitrophenol from the incubation system with the method of Bligh and Dyer (1959), and comparison against a standard curve of p-nitrophenol (Galliard, 1971a).

Naphthol released by hydrolysis of 2-naphthyl esters can be determined by coupling with tetra-azotized O-dianisidine, thus producing a pigment (Galliard, 1971a).

2.4.2 Assay of the Liberated Fatty Acids

Procedures involving the determination of the liberated fatty acids are the most numerous in the reports concerning lipolytic acyl hydrolases. In many cases the freed fatty acids may be measured

directly by a titration (Dole and Meinertz, 1960). In recent years however, there has been a definite preference towards the use of radioactivity-labelled lipid substrates, which offer the greatest sensitivity (Brockerhoff et al., 1970). In a typical procedure, enzyme, buffer and the labelled lipid substrate such as 2-[1-^{14}C] oleyl-phosphatidylethanolamine, are incubated for a set period of time. The reaction is stopped with the addition of methanol and the products of hydrolysis are extracted by the method of Bligh and Dyer (1959). Radioactive lipids are separated by thin-layer chromatography on silica gel plates and developed using the appropriate solvent. After staining the chromatograms with I_2 vapor, the silica gel containing radioactive lipid is scraped into vials containing scintillation fluid and the radioactivity determined in a scintillation counting system (Franson et al., 1978).

A fluorometric method was used to study the lipid acyl hydrolase activity in potato tubers by Hasson and Laties (1976a). 4-Methylumbelliferone, N-methylindoxyl and fluorescein fluoresce, wheareas their respective fatty acid esters do not. Hydrolytic activity was determined by measurement of the fluorescence intensity following the addition of fluorogenic ester to a volume of buffered enzyme. Fluorescence was measured with a Farrand Ratio Fluorometer with appropriate excitation and emission wavelengths.

Various colorimetric methods have also been employed. Most of the reported methods depend on the formation of a copper soap of the freed fatty acid, its extraction into an organic solvent, and subsequent estimation of copper (Baker, 1961; Duncombe, 1963). Anderson and McCarty (1972), describe a rapid and sensitive method

using rhodamine 6G. Mahadevan et al., (1969) describe a modification to the procedure of Duncombe (1963), which increases the sensitivity 4 to 6 times, thus making the method more suitable for assaying lipolytic enzymes with low specific activities.

Finally, there is a method that indirectly measures the freed fatty acids by measuring the subsequent decrease of ester groups in the lipid substrates (Snyder and Stephens, 1959), modified by Renkonen (1961). It is an extremely simple yet highly sensitive and accurate assay based on a hydroxylaminolysis in which an ester reacts with alkaline hydroxylamine to form a hydroxamic acid, which in turn forms a purple iron-chelate complex in the presence of acid ferric perchlorate. This method has been used with acyl hydrolases from a selection of plant sources including potato tubers (Galliard, 1970; 1971a, Shepard and Pitt, 1976b) and cucumber (Galliard et al., 1976).

2.5 Purification

Many difficulties have been encountered in attempts to isolate and purify lipolytic acyl hydrolases. This is due mainly to the association of the enzyme to tightly-bound lipids and/or a major protein fraction, which require severe conditions for separation which leads to extensive loss of enzymic activity. This is true, although to a lesser extent, with animal sources. The relative ease of purifying animal phospholipases is due to the fact that acyl hydrolase activity is proportionately higher in animal tissues. Phospholipase A is present in high concentrations in the venom of many domestic and foreign snakes, and has been detected in bee and scorpion venoms and in many different mammalian tissues, with particularly

significant concentrations in pancreas and small intestine (Wells and Hanahan, 1969). Purification procedures usually begin with dehydration and delipidation by means of acetone or ammonium sulfate fractionation, although the preferred purification procedure of Crotalus adamanteus venom begins with a lyophilization step to dehydrate the venom (Wells and Hanahan, 1969). C. adamanteus venom contains two proteins with phospholipase A activity (Saito and Hanahan, 1962). One gram of lyophilized venom is extracted into a solution of 0.1 M NaCl, 50 mM Tris and 1 mM EDTA, pH 8.0, and subjected to a series of chromatographic separations. Sephadex G-100 chromatography is the first step, followed by chromatography on DEAE - Cellulose and SE - Sephadex. Each subsequent chromatography is preceded by a dialysis step to concentrate the previous eluant. The two proteins mentioned above can now be separated clearly by ion exchange chromatography and disc gel electrophoresis. This procedure gives a purification of approximately 20-fold.

A similar pattern of purification steps is presented by van den Bosch et al. (1973), who purified a lysophospholipase from beef pancreas. A cell-free homogenate was first subjected to acid (pH 4.0) precipitation, the supernatant from which was treated with ammonium sulfate; 20% n-butanol. This gave a precipitate which was extracted into an acetate buffer pH 4.0. The extract was purified by chromatography with SE - Sephadex - C50 followed by DEAE - Cellulose, a second SE - Sephadex - C50, and finally DEAE - Sephadex - A50. This entire procedure gave a final purification of 164-fold.

In microorganisms, phospholipases are located chiefly in the membrane (Bernard et al., 1972). The cells must therefore be disrupted to extract the desired enzyme. This has been done by

the application of lysozyme [EC 3.2.1.17], which hydrolyses the cell-wall substance of certain bacteria (Doi and Nojima, 1973). More recently, Nishijima et al., (1977), disrupted frozen wet cells of Escherichia coli at a pressure of 400 kg/cm^2 in a French press. This was followed by solubilization with sodium dodecylsulfate and butan-1-ol, acetate precipitation, acetone fractionation and extraction of lipid with butan-1-ol. The resulting solution was then subjected to column chromatography with Sephadex G-100 and DEAE - cellulose giving a final purification of nearly 1700-fold.

Comparatively less success has been obtained with purification of lipolytic acyl hydrolases from plant sources. The first attempts in purification were by Galliard (1971a) with potato tubers. Using acetone precipitation, a series of chromatographic steps, and a final suspension in ammonium sulfate, he obtained a very low purification factor of 5.4. This apparently poor degree of purification was due to a close association, throughout the purification procedures, of enzyme activity with a major protein component and was not considered to be caused by loss of enzyme activity during purification. When using this preparation, activity was noted with a very diversified range of substrates. It was argued that if a single enzyme was not responsible for the deacylation of the different lipid classes described, then the enzymes involved were similar with respect to subcellular location, molecular size and charge, and their behavior with substrates, inhibitors and detergents. However, it was also observed that three distinct bands of esterase activity were separated on gel electrophoresis, thus raising the possibility of isoenzymic forms of the lipolytic acyl hydrolase.

Shepard and Pitt (1976b), used a series of purification steps including gel filtration, ion-exchange chromatography and isoelectric focusing interspersed with many dialysis and lyophilization steps, to partially separate enzyme activities, again in potato tubers. Phospholipase and galactolipase activity was separated from lipolytic acyl hydrolase activity (p-nitrophenyl palmitate as substrate). However, they were forced to conclude that it was difficult to state whether the purification procedures used separated different enzymes or whether structural modifications resulted to portions of a single, non-specific enzyme, causing the generation of sub-units of altered isoelectric values and differing substrate specificities.

Hasson and Laties (1976a), separated three distinct lipolytic enzymes from potato tubers. Based on substrate specificities, they determined a lipase, a phospho- and galactolipase and an esterase. The purification steps involved included ammonium sulphate fractionation followed by filtration with Sephadex G-200 and DEAE - cellulose. They obtained an overall purification of 139-fold for the lipase, 56-fold for the phospho- galactolipase and 20-fold for the esterase.

A pure lipid acyl hydrolase was prepared from potato tubers by Hirayama et al. (1975) using acetone precipitation, Sephadex G-100 chromatography, DEAE - Sephadex, A-50 chromatography and isoelectric focusing. The sequence of the purification procedures effected 380- and 350-fold increases in specific activity of galactolipase and phospholipase, respectively. A similar purification procedure was used to separate a lipase, phospholipase, and galactolipase from rice bran (Hirayama and Matsuda, 1975) and rice endosperm (Matsuda and Hirayama, 1975). Subsequent work with the enzyme isolated from

potato tuber showed that it could dissociate into two or three proteins depending on whether 8M urea or 1% sodium dodecyl sulfate was added. The reassociation of the subunit proteins with partial recovery of the enzyme activities was attained by dialysis in the presence of Ca^{2+} (Matsuda et al., 1977).

2.5.1 Isoenzymes of Lipolytic Acyl Hydrolases

The preliminary work by Galliard (1971a), indicated that isoenzymic forms of the acyl hydrolase might be present. The presence of multiple forms of esterases has been demonstrated in a wide variety of animal tissues (Paul and Fottrell, 1961), pea and green bean (Veerabhadrappu and Montgomery, 1971) and in potato tuber (Schwartz et al., 1964), using various electrophoretic techniques. On the basis of genetic studies with potato tubers, Desborough and Peloquin (1967) postulated that the esterase isoenzymes are tetramers in which the assembly of monomeric subunits is under genetic control. The presence of isoenzymic forms of lipolytic acyl hydrolase in potato was reported by Galliard and Dennis (1974b) and they were shown to be active in the formation of wax esters (Dennis and Galliard, 1974). Isoenzymes of the phospholipase from Naja naja venom were reported by Shiloah et al. (1973a).

2.6 Criteria of Purity

There is no direct way of demonstrating that an enzyme is pure. A decision in favor of homogeneity lies in the negative evidence of heterogeneity. In such a case the confidence is proportional to the number of techniques tried. It is important to run each test under different conditions of pH, ionic strength, etc. It is beneficial to know the degree of purification attained with each purification

step in order to determine when an enzyme solution is pure or if further purification procedures are necessary. Two classical tests employed are electrophoresis in polyacrylamide gels and isoelectric focussing in polyacrylamide gels. The electrophoresis techniques allow the study of isoenzymic forms of enzyme activity, as well as the degree of purification attained, while isoelectric focusing demonstrates the purity of the enzyme and isoelectric point of the protein involved.

Highly-purified lipolytic acyl hydrolases from a variety of plant, animal and microbial sources have been shown to contain only a single band of activity on polyacrylamide gel disc electrophoresis (Hirayama et al., 1975; Nishijima et al., 1977; Franson et al., 1978). Isoenzymic patterns have been demonstrated for a plant acyl hydrolase (Galliard and Dennis, 1974b), and an animal phospholipase (Shiloah et al., 1973a), indicating that activity is not universally confined to one protein fraction.

Isoelectric focusing was used by Shepard and Pitt (1976b), to demonstrate two enzyme fractions with differing substrate specificities, one a phospholipase (pI 4.24), the other an acyl hydrolase active with p-nitrophenyl esters (pI 7.29). The isoelectric point of beef pancreas lysophospholipase was estimated between 5.0 and 6.0 (van den Bosch et al., 1973).

Other techniques have also been used to determine the purity of a preparation. Ultracentrifugation tests sedimentation properties of proteins in terms of size and shape in high centrifugal fields. Heterogeneity would be indicated by the presence of more than one sedimentation peak or by a non-uniform distribution of peaks. The enzyme preparation of Hirayama et al. (1975) was

homogeneous with a symmetric sedimenting boundary. The purity of a carboxyl ester hydrolase from human pancreatic juice was tested by the technique of immunoelectrophoresis which gave a single precipitin line against an antiserum to the total proteins of human pancreatic juice (Lombardo et al., 1978). This result was substantiated by ultracentrifugation.

2.7 Molecular Weight of Lipolytic Acyl Hydrolases

Molecular weights of the various acyl hydrolases reported in the literature are usually determined by gel filtration. Sephadex columns are calibrated by applying a mixture of proteins of known molecular weights. A linear relationship is obtained between elution volume and log (molecular weight). A protein of unknown molecular weight can be run on the column, and its molecular weight determined from its corresponding elution volume. Meaningful results can only be obtained when pure enzyme preparations are used, therefore the rather high figure of 107,000 reported by Galliard (1971a) must be considered as a rough estimate due to the low purification of his potato tuber enzyme. Later studies obtained enzyme preparations from potato tubers that were highly purified in comparison. Hasson and Laties (1976a) separated three lipolytic enzymes: a lipase, M.W. 77,000; a phospho-galactolipase, M.W. 63,000; and an esterase, M.W. 23,000. A molecular weight of 70,000 was reported for the potato acyl hydrolase of Hirayama et al. (1975). Three distinct enzymes, a lipase, a phospholipase and a galactolipase purified from rice bran were found to have similar molecular weights of 40,000 (Hirayama and Matsuda, 1975).

Phospholipase A from Escherichia coli was found to have a

molecular weight of 28,000 (Nishijima et al., 1977). DeHaas et al. (1968) have purified phospholipase A from porcine pancreas and found it to have a molecular weight of 14,000. The phospholipase A_2 from inflammatory exudate from rabbit has a molecular weight of 14,800 (Franson et al., 1978) while the beef pancreas lysophospholipase was determined to be between 63,000 and 67,000 (van den Bosch et al., 1973).

2.8 Chemical Properties of Lipolytic Acyl Hydrolases

2.8.1 Substrate Concentration

The affinity of an enzyme for a given substrate is measured by the Michaelis-Menten constant (Km). The Km constant is calculated using a Lineweaver-Burk plot, and indicates the substrate concentration at which one-half of the enzyme reaction maximum velocity is reached. Because of the diversity of data regarding substrates employed and the type of enzyme preparations used, it is difficult to report a concise representation of the literature. However, by limiting to the most relevant substrates, some useful generalizations may be made. Using lecithin as substrate the following Km values were reported: 1.7 mM for potato lipid acyl hydrolase (Hirayama et al., 1975), 0.22 mM for phospholipase A from Escherichia coli (Doi et al., 1972) and 0.8 mM for phospholipase A_1 from rat brain (Gatt et al., 1968). Galliard (1971a) assayed a potato lipid acyl hydrolase with a number of lipid substrates and obtained Km values of 0.4 mM for galactosyl diglyceride, 0.7 mM for mono-olein, 0.5 mM for p-nitrophenyl palmitate, 0.7 mM for p-nitrophenyl stearate and 2.0 mM for p-nitrophenyl acetate.

2.8.2 Effect of pH

The effect of pH on the rate of hydrolysis by lipolytic acyl hydrolases varies dramatically depending on the source of the enzyme. Optimum pH values have been reported that cover the range of 5.0 - 9.5, excluding the 4.0 pH optimum reported for phospholipase A_1 from rat brain (Gatt et al., 1968). Even with the same enzyme source considerable variation in pH optima occurs. Using enzymes from potato tubers, Galliard (1970) and Shepard and Pitt (1976b) reported 5.0 - 5.6 as the optimum pH, while 7.0 - 7.5 was reported by Hasson and Laties (1976a) and 8.5 reported by Hirayama et al. (1975). Galliard et al. (1976) reported lecithin hydrolysis in cucumber to be maximal at pH 5.0 - 5.5. Phospholipases of Escherichia coli have pH optima of 8.0 - 8.5 (Doi and Nojima, 1973; Nishijima et al., 1977), while Clostridium welchii has an optimum pH of 7.0 - 8.0 (Nakamura et al., 1969). Animal lipolytic acyl hydrolases have pH optima spanning the wide range listed above and often demonstrate bimodal or trimodal pH optima (Franson et al., 1978; Wassef et al., 1978).

2.8.3 Temperature

Lipolytic acyl hydrolases are active over a wide temperature range (2° - 50°C) with optimal temperatures in the range of 25 - 37°C depending on the enzyme source. Several studies have investigated the stability of these enzymes at elevated temperatures and have shown them to be relatively heat-labile. The lipid acyl hydrolase of Galliard (1971a) was reported to be rapidly inactivated at 100°C and showed an approximate 50% loss of activity towards p-nitrophenyl palmitate after treatment for 3 minutes at 60°C.

2.8.4 Action of Activators

Although it is desirable to perform kinetic studies in simple systems, it is often necessary to use substances that will provide reasonable reaction rates, and in some cases any reaction at all. The surfactant Triton X-100 has had extensive use in the study of lipid acyl hydrolases, and is thought to enhance deacylation by altering the physical form of the lipid substrate making the acyl ester bond more accessible to enzyme attack (Dennis, 1974). Galliard (1971a) found that hydrolysis of lecithin could not be demonstrated in the absence of Triton X-100, but later reported that free fatty acids could stimulate lecithin hydrolysis in its absence (Galliard, 1971b). Electron microscope studies suggested that free fatty acids altered the physical state of the lipid substrate in a fashion similar to that of Triton X-100. This requirement for free fatty acids was also reported by Subbaiah and Ganguly (1970). Another known effect of Triton X-100 is to shift the pH optimum of the enzyme preparations, from 5.5 to 7.5 in the case of potato tuber lipid acyl hydrolase (Galliard, 1971a). Other surface-active agents such as bile salts have had relatively less application.

Calcium chloride has been used as an activator in many studies, with a strong requirement for the calcium ion demonstrated in many of these reports. The activation of lipolysis by calcium chloride has been known for a long time (Willstätter et al., 1923), and has been assumed to result from the removal of unionized fatty acids from the interface through the formation of insoluble calcium soaps (Benzonana and Desnuelle, 1968). Other divalent cations such as magnesium, barium and strontium have been used as activators, but their effect is negligible when compared to calcium stimulation

(Nishijima et al., 1977).

2.8.5 Inhibitors of Lipolytic Acyl Hydrolases

Lipolytic acyl hydrolases are inhibited by heavy metals such as Cu^{2+}, Zn^{2+} and Fe^{3+} (Nishijima et al., 1977), as are many hydrolytic enzymes. At concentrations of 5 mM, they were found to almost completely inhibit the hydrolysis of phosphatidylethanolamine. The chelating agent EDTA is a potent inhibitor, acting presumably, by immobilizing required ions such as Ca^{2+} (Franson et al., 1978).

Use has been made of various reagents specific for chemical modifications in order to determine the residues in the active site necessary for enzyme activity. Phospholipase from potato lost no activity when treated with a high concentration of p-chloromercuri-benzoate indicating that a sulfohydroxyl group is not involved in the active site (Hirayama et al., 1975). Additional work indicated that histidine and serine residues were important to enzyme activity, and that a tyrosine residue might be involved in the activity as an accessory component.

3. MATERIALS

3.1 Enzyme Source

Lipolytic Acyl Hydrolase was isolated from fababeans (Vicia faba L. var. minor cv. Diana) which were obtained from the Department of Plant Science, University of Manitoba. The beans were harvested in 1976 and subsequently stored at room temperature. Crude hog pancreatic lipase (EC 3.1.1.3) was purchased from the Sigma Chemical Co.

3.2 Chemicals

Hydroxylamine hydrochloride, 70% perchloric acid, chloroform, methanol and Phenol Reagent Solution 2N were obtained from Fisher Scientific Co., New Jersey. Methyl palmitate was purchased from Eastman Kodak Co., Rochester, New York, while BDH Chemicals, Toronto, supplied bovine serum albumin. Triton X-100, p-nitrophenol, p-nitrophenyl palmitate, p-nitrophenyl stearate, p-nitrophenyl laurate, p-nitrophenyl mysistate, p-nitrophenyl acetate, L- α- phosphatidic acid and L- α- phosphatidyl choline were purchased from Sigma Chemical Co., St. Louis, Missouri. Ferric perchlorate was supplied by Matheson, Coleman and Bell, Norwood, Ohio. while 1,2 dilinolein was obtained from Nu - Chek - Prep Inc., Elysian, Minnesota. All other chemicals and reagents were of analytical grade.

4. METHODS

4.1 Assay Procedures

4.1.1 p-nitrophenyl Ester Hydrolase Assay

The assay used to study the hydrolysis of p-nitrophenyl esters was essentially that of Galliard (1971a). Lipolytic acyl hydrolase activity was determined by measuring the release of p-nitrophenol from the p-nitrophenyl-fatty acid esters. The initial velocity of lipolysis was followed with a Unicam SP 800B Ultraviolet recording Spectrophotometer fitted with a Unicam SP 825 series 2 Programme Controller and a Unicam Sp 850 Scale Expander. A constant assay temperature (25°C) was attained with a Heto Controlled Temperature Circulating Water bath which was connected to the water jacket of the cuvette holder. A continuous readout was recorded by a Unicam SP 20 external recorder. The emulsified substrate (2.0 mM p-nitrophenyl-fatty acid ester, stabilized with Triton X-100, 4.0 mg/ml, by continuous agitation on a Vortex mixer for 2-5 minutes) was introduced into the reaction cuvette along with appropriate buffer to ensure a final assay volume of 3.0 ml. Sufficient enzyme solution was then added to the cuvette, mixed, and the assay allowed to proceed monitoring the A_{400} against a substrate-buffer blank. Total assay time was ten minutes. The initial reaction velocity (first minute of assay) was determined by calculating the increase in p-nitrophenol concentration. This was accomplished by relating the change of A_{400} to a standard curve for p-nitrophenol measured at the pH of incubation. One unit of lipolytic acyl hydrolase (LAH) activity is equal to one micromole of p-nitrophenol released per minute at 25°C, and is calculated from the equation:

$$\text{LAH ACTIVITY} = \frac{\left([\text{p-nitrophenol}]_{1\ \text{min}} - [\text{p-nitrophenol}]_{0\ \text{min}} \right) \times 3.0\ \text{ml}}{\text{mg protein used}}$$

Controls were carried out to check nonenzymic hydrolysis of the substrate, which was found to be negligible in the pH range of 6.0 to 9.0.

4.1.2 Phospholipase Assay

The phospholipase assay was a modification (Renkonen, 1961) of the spectrophotometric determination of ester groups of Snyder and Stephens (1959). The rate of hydrolysis was determined by measuring the decrease of acyl ester content at the end of a defined incubation period (10 or 30 minutes depending on the particular substrate assayed) at 25°C. The assay was carried out in standard 18 mm X 150 mm test tubes. 0.05 - 1.00 micromole of emulsified substrate (0.1 - 2.0 microequivalents of acyl ester) was placed in the test tube. Triton X-100 (4 mg/ml) was used to stabilize the emulsion (formed by high-speed mixing on a vortex mixer for 2-3 minutes). Sufficient buffer was added to each test tube to ensure a final assay volume of 2.4 ml, after which 1.0 ml of enzyme solution was added. The test tubes were mixed thoroughly after addition of both buffer and enzyme solution. Appropriate substrate and enzyme solution blanks were included. The reaction was terminated by placing the test tubes in a boiling water bath. After cooling, 3.0 ml chloroform and 6.0 ml methanol were added to each test tube (total volume 11.4 ml) to form the uniphasic mixture of Bligh and Dyer (1959). Each test tube was mixed on a vortex mixer for 1 minute, and allowed to stand for a further 30 minutes. At this point 3.0 ml chloroform and 0.2 M acetate buffer, pH 4.0 (3.0 ml) were added; the acid buffer was necessary to prevent

the formation of emulsions caused by free fatty acids liberated during lipolysis. The tubes were shaken and allowed to stand until complete separation of the chloroform and aqueous methanol phases was achieved. The upper layer of the mixture was drawn off by means of an aspirator, and the test tubes containing the chloroform layers were placed in a vacuum and evaporated to dryness. When drying was complete, 1.0 ml of alkaline hydroxylamine reagent was added to each tube, shaken, and placed in a water bath (65°C) for 2 minutes. After cooling for 5 minutes, 3.0 ml of ferric perchlorate reagent were added to each test tube and mixed thoroughly. The purple color was allowed to develop for 30 minutes. The A_{530} for each sample was then determined using a Unicam SP 600 series 2 spectrophotometer. By relating the A_{530} values to a standard curve relating absorbance to known amounts of ester (methyl palmitate as substrate), the number of microequivalents of ester remaining after lipolysis could be determined. The purple color was stable for approximately 1 hour after final color development. Experiments to determine the acyl ester content of the enzyme solution showed that a significant proportion (approximately 1.1 microequivalents of acyl ester per ml enzyme solution) of acyl ester in the ester determination was due to the lipid fraction of the enzyme solution. Correcting for this, one unit of phospholipase (PPL) activity is equal to one microequivalent of ester hydrolyzed in ten minutes at 25°C, and is equal to the equation:

$$\text{PPL ACTIVITY} = \frac{(P + S) - H}{\text{mg. protein used}}, \text{ where:}$$

P = microequivalents of ester added in enzyme solution.

S = microequivalents of substrate ester added; and

H = total microequivalents of ester remaining after hydrolysis.

4.1.2.1 Reagents

(1) Stock Ferric Perchlorate : 5.0 gm ferric perchlorate were dissolved in 10 ml 70% $HClO_4$ and 10 ml distilled water, and then diluted to 100 ml with cold (0°C) absolute ethanol. This solution was stored at 0°C.

(2) Reagent Ferric Perchlorate : 4 ml stock ferric perchlorate and 3 ml 70% $HClO_4$ were diluted to 100 ml with cold absolute ethanol. This reagent was prepared daily.

(3) 4% Ethanolic Hydroxylamine : 2.0 gm hydroxylamine hydrochloride were dissolved in 2.5 ml water and diluted to 50 ml with absolute ethanol. This solution was stored at 0°C when not in use.

(4) 8% Ethanolic NaOH : 4.0 gm NaOH were dissolved in 2.5 ml water, and diluted to 50 ml with absolute ethanol. This solution was prepared fresh daily.

(5) Alkaline Hydroxylamine Reagent : Equal volumes of a 4% ethanolic hydroxylamine solution and of an 8% ethanolic NaOH solution were mixed in a stoppered cylinder. The NaCl was separated by centrifugation (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge, 12,000 Xg, 15 min. 20°C) and the supernatant was decanted for use.

4.2 Protein Determination

Protein was determined by the method of Lowry et al., (1951). Commercial Folin-Ciocalteu reagent was diluted twice immediately before use. Crystalline bovine serum albumin served as the standard for the calibration curves. The absorbance of the blue solutions was determined on a Unicam SP 600 spectrophotometer at 500 nm.

4.3 Preparation of Acetone Powder

Whole fababeans were dehulled and ground to a powder in a Wiley Mill. The powder was extracted in 3 volumes of acetone (-20°C) by high-speed blending in a Waring blender. The mixture was immediately filtered with suction through Whatman No. 4 filter paper. A light-colored, low-density solid decanted with the acetone, and a small amount of dark fibrous residue remained in the blender. The off-white precipitate was washed with 3 successive portions of acetone, air-dried and crushed. The resulting powder was stored at 4°C.

4.4 Partial Purification

Both p-nitrophenyl ester hydrolase and phospholipase activity were determined in a crude extract of the acetone powder. Ten gm of acetone powder were blended with 10 volumes of 0.1 M Diethanolamine-HCl buffer, pH 8.5, or with 10 volumes of 0.1M Acetate buffer, pH 5.6 (for p-nitrophenyl ester hydrolase and phospholipase assays respectively). The homogenate was allowed to stand for 15 minutes, and was then centrifuged twice at 12,000 X g for 30 minutes at 0°C (Sorvall RC2-B centrifuge). The supernatant was retained for enzyme assay, and subsequently stored at 4°C under toluene.

An initial attempt was made to purify the crude extract using acetone fractionation. The crude extract (4°C) was concentrated by the addition of precooled (-26°C) acetone, the temperature of the mixture not being allowed to rise above -15°C. The final acetone concentration was first brought to 30% whereupon the mixture was immediately centrifuged at 12,000 X g for 10 min at -20°C. The precipitate was a thick oily residue that would not dissolve in water, and did so very slowly in chloroform. It was concluded that

this was the lipid or lipoprotein fraction of the acetone powder. The supernatant was then brought to a final acetone concentration of 60% and then centrifuged at 12,000 X g for 10 min at -20°C. The precipitate was collected, washed with acetone, centrifuged, and freeze-dried (Virtis 10 - 146 MP - BA Freeze-Mobile). The white powder was dissolved in buffer and assayed for lipolytic acyl hydrolase activity with p-nitrophenyl palmitate. Although considerable activity was detected, the specific activity of this preparation was lower than that of the crude extract. This was concluded to be due to the separation of the lipid component from the acetone protein fraction. Based on this assumption and the time factor, it was decided that no further purification attempts would be made. The crude extract was used for the kinetic studies.

4.5 Determination of Michaelis Constant

The Michaelis constant (Km) was determined for a number of p-nitrophenyl-fatty acid esters and phospholipids using the appropriate assay. The effect of enzyme concentration on the reaction was determined prior to the Michaelis-Menten studies, resulting in a linear relationship up to a protein concentration of 12 mg/ml. In tests to determine Km values, protein concentrations of 8-10 mg/ml were used.

The initial reaction velocities of various substrate concentrations (0.01 - 1.5 mM) were determined and plotted against their respective substrate concentrations. All trials were carried out in triplicate. A reciprocal plot of velocity against substrate concentration was used to determine the Km (Lineweaver and Burk, 1934).

4.6 Effect of pH

In order to determine the optimum pH of fababean lipolytic acyl hydrolase and phospholipase, saturated substrate mixtures (0.67 mM p-nitrophenyl palmitate or 0.64 micromole of phosphatidylcholine) were assayed for rates of hydrolysis using a fixed protein concentration (8 mg/ml and 10 mg/ml respectively). The pH range was obtained by extracting portions of the acetone powder directly into buffers of a particular pH. Each appropriate buffer was also used at the time of incubation to ensure the proper assay volume (2.4 ml for phospholipase assays, 3.0 ml for p-nitrophenyl ester hydrolase assays). The buffers used were: 0.1M acetate buffer for pH 4.5 - 5.6; 0.1M phosphate buffer for pH 6.0 - 8.0 and a diethanolamine-HCl buffer for pH 8.5 - 9.5 Studies were undertaken to determine the effect of non-enzymic hydrolysis of p-nitrophenyl palmitate at each pH of assay. This effect was found to be negligible in the pH range 6.0 - 9.0 Non-enzymic hydrolysis of the lecithin substrate was not observable in the pH range studied (4.5 - 8.5).

4.7 Effect of Temperature

In order to determine the optimum temperature of fababean lipolytic acyl hydrolase, assays were carried out at various temperatures from 25 - 55°C using p-nitrophenyl palmitate as substrate. The temperature was adjusted by means of the temperature control on the Heto circulating water bath. The actual temperature inside the assay cuvettes was also monitored.

The heat stability of the enzyme was determined by exposing aliquots of enzyme solution (20 ml) in the absence of substrate, to different temperatures. Aliquots were removed at one or two-minutes intervals

and directly assayed for residual activity at 25°C and pH 7.5.

4.8 Effect of Triton X-100 Concentration

In order to determine the effect of Triton X-100 concentration on the activity of fababean lipolytic acyl hydrolase, a series of experiments was performed in which the amount of Triton X-100 used to stabilize the lipid substrate was varied. Emulsions of p-nitrophenyl palmitate and phosphatidyl choline (for the p-nitrophenyl ester hydrolase and phospholipase assays respectively) were prepared with Triton X-100 concentrations in the range of 2.0 - 16.0 mg/ml. Standard assay procedures were followed for both assays.

4.9 Effect of Activators and Inhibitors

A series of experiments was performed to determine the effects of various compounds on the rate of activity of fababean lipolytic acyl hydrolase. Calcium chloride was tested as an activator in both the p-nitrophenyl esters hydrolase and phospholipase assays. The inhibitor tested, again in both assays, was mercuric chloride. Inhibition or activation was calculated after determination of the rate of hydrolysis of either p-nitrophenyl palmitate (0.67 mM) or phosphatidyl choline (0.13 mM) in the presence and absence of the various compounds at different levels of concentration. Trials were run in triplicate and all other conditions followed the standard assay procedures.

5. RESULTS

5.1 Effect of Enzyme Concentration

The relationship between the amount of enzyme and the reaction rate was investigated in order to determine protein linearity. The effect of enzyme concentration on the reaction rate is shown in Figure 2, which demonstrates the initial velocity being strictly a linear function of the amount of enzyme present up to a protein concentration of 12 mg./ml. A standard protein concentration of 10 mg./ml. was used for later enzyme studies with the phospholipase assay, while for the p-nitrophenyl ester hydrolase assay a standard protein concentration of 8 mg./ml. was used. The crude fababean extract used in all enzyme studies yielded an average protein concentration of 24 mg./ml.

5.2 Effect of Substrate Concentration and Calculation of Michaelis Constant

5.2.1 p-nitrophenyl Ester Substrates

The initial velocities in the presence of various concentrations of each of the p-nitrophenyl ester substrates (p-nitrophenyl acetate; -laurate; -myristate; -palmitate and -stearate) were determined and plotted against their respective substrate concentrations. This was carried out for each substrate at two pH values (7.5 and 8.5), with the exception that the assay could not be carried out at pH 7.5 with p-nitrophenyl stearate. The p-nitrophenyl stearate emulsion was very unstable and would break down when the pH was adjusted to 7.5. The substrates are expressed as molar concentrations, but as lipolytic acyl hydrolase-catalyzed hydrolysis occurs only at the interface of a heterogeneous system, substrate

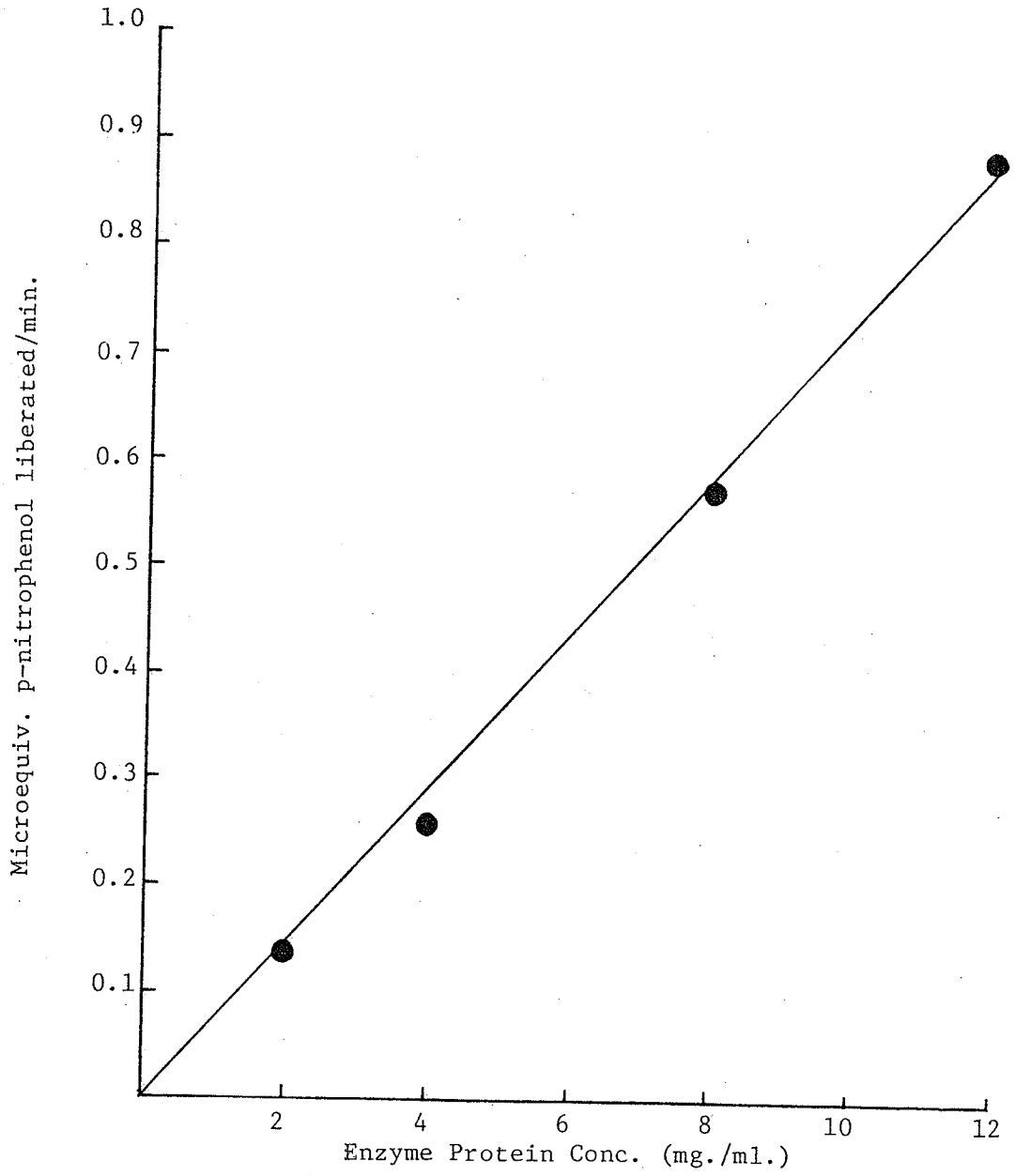


Figure 2. Effect of Enzyme Concentration on Fababean Lipolytic Acyl Hydrolase Activity. p-nitrophenyl Palmitate Substrate.

concentration is not strictly governed by the Michaelis rule relating the rate of the reaction to the molar concentration of the substrate in homogeneous systems. In this case substrate concentration means the "emulsion" concentration, and as such is expressed as a molar term.

The effect of substrate concentration on lipolytic acyl hydrolase activity is presented for p-nitrophenyl palmitate; -stearate; -myristate; - laurate and -acetate in Figures 3, 5, 7, 9 and 11 respectively. The data are shown as rectangular hyperboles with marked deviations occurring at higher substrate concentrations. The lipolytic acyl hydrolase activity was found to be inhibited by high substrate concentrations. For all the substrates containing long-chain fatty acids, inhibition occurred at substrate concentrations of 0.34 mM in the case of p-nitrophenyl laurate (pH 8.5) to 1.0 mM in the case of p-nitrophenyl palmitate (pH 8.5). The hydrolysis of p-nitrophenyl acetate, however, was linear with respect to substrate concentrations up to at least 0.7 mM, suggesting that the substrate inhibition may be due to interaction between enzyme and the long-chain fatty acid components of lipids.

At sub-optimum substrate concentrations the reactions followed the reciprocal Lineweaver-Burk relationship (Figures 4,6, 8, 10 and 12) but gave marked deviations at higher concentrations. Extrapolation of the linear portions of the Lineweaver-Burk plots enabled values to be calculated for the apparent Km with each substrate. Calculated values of Km and Vmax for each substrate assayed are summarized in Table 1.

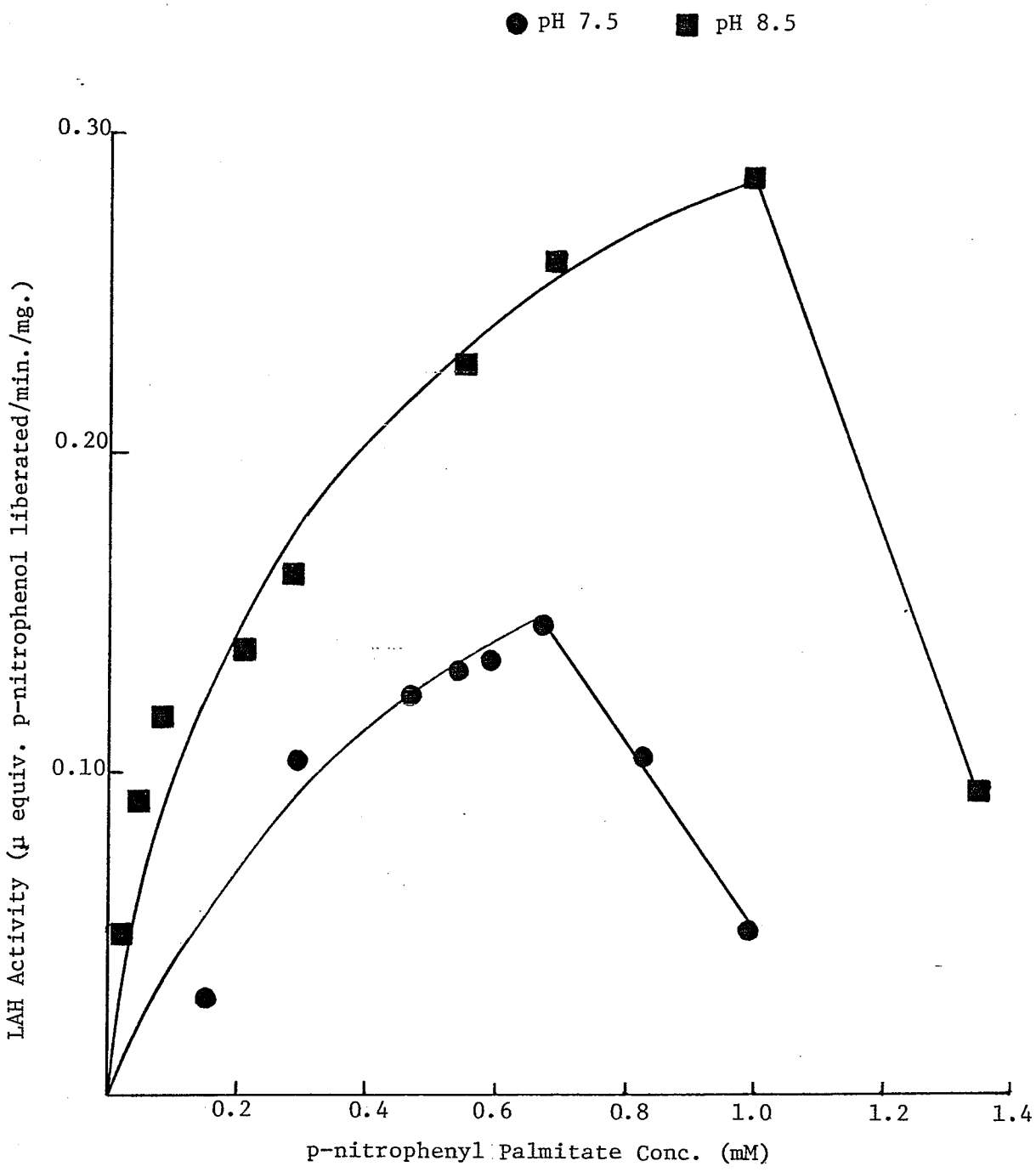


Figure 3. Effect of p-Nitrophenyl Palmitate Concentration on Fababean Lipolytic Acyl Hydrolase.

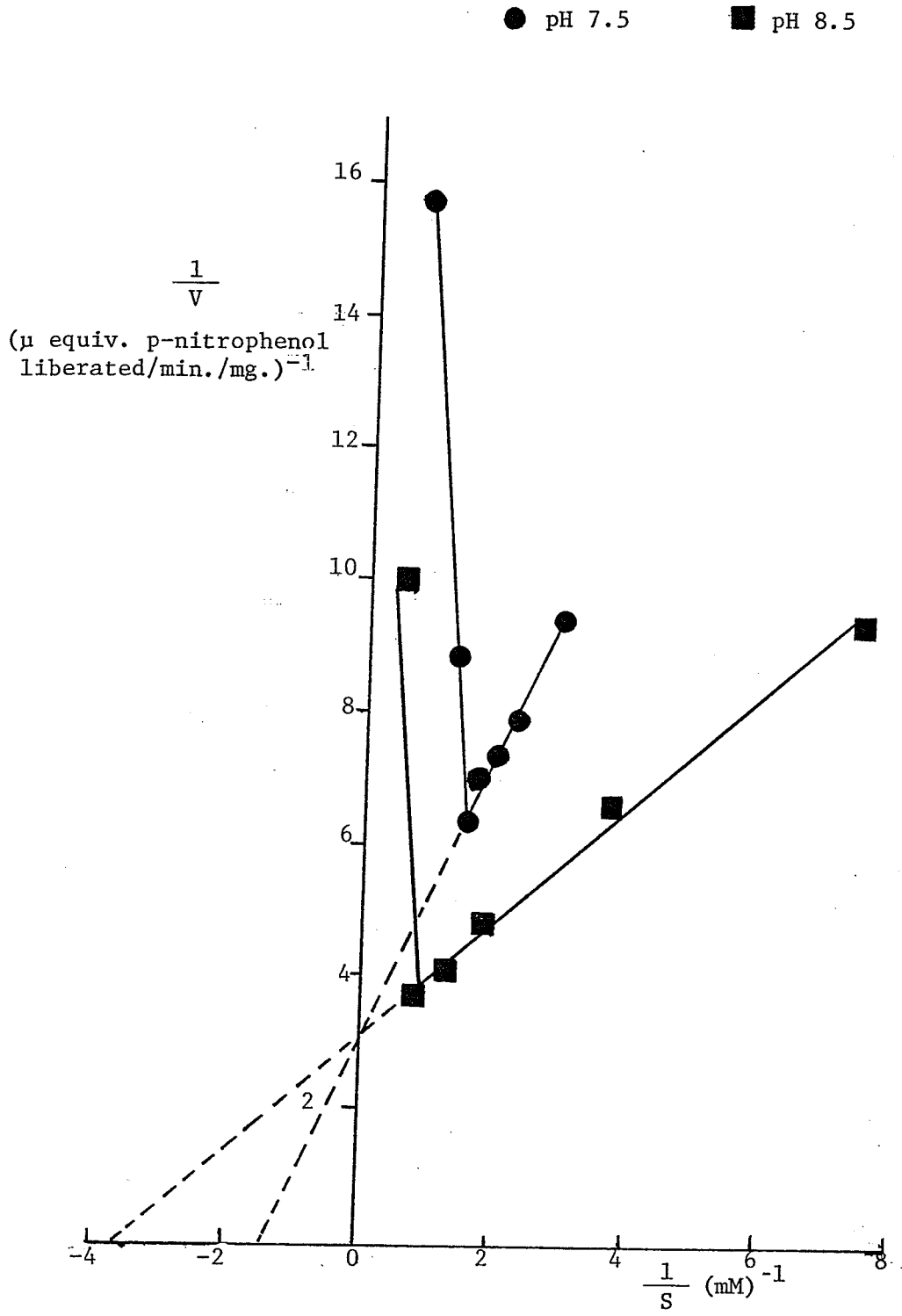


Figure 4. Lineweaver-Burk Plot of the Effect of p-Nitrophenyl Palmitate Concentration on Fababean Lipolytic Acyl Hydrolase Activity.

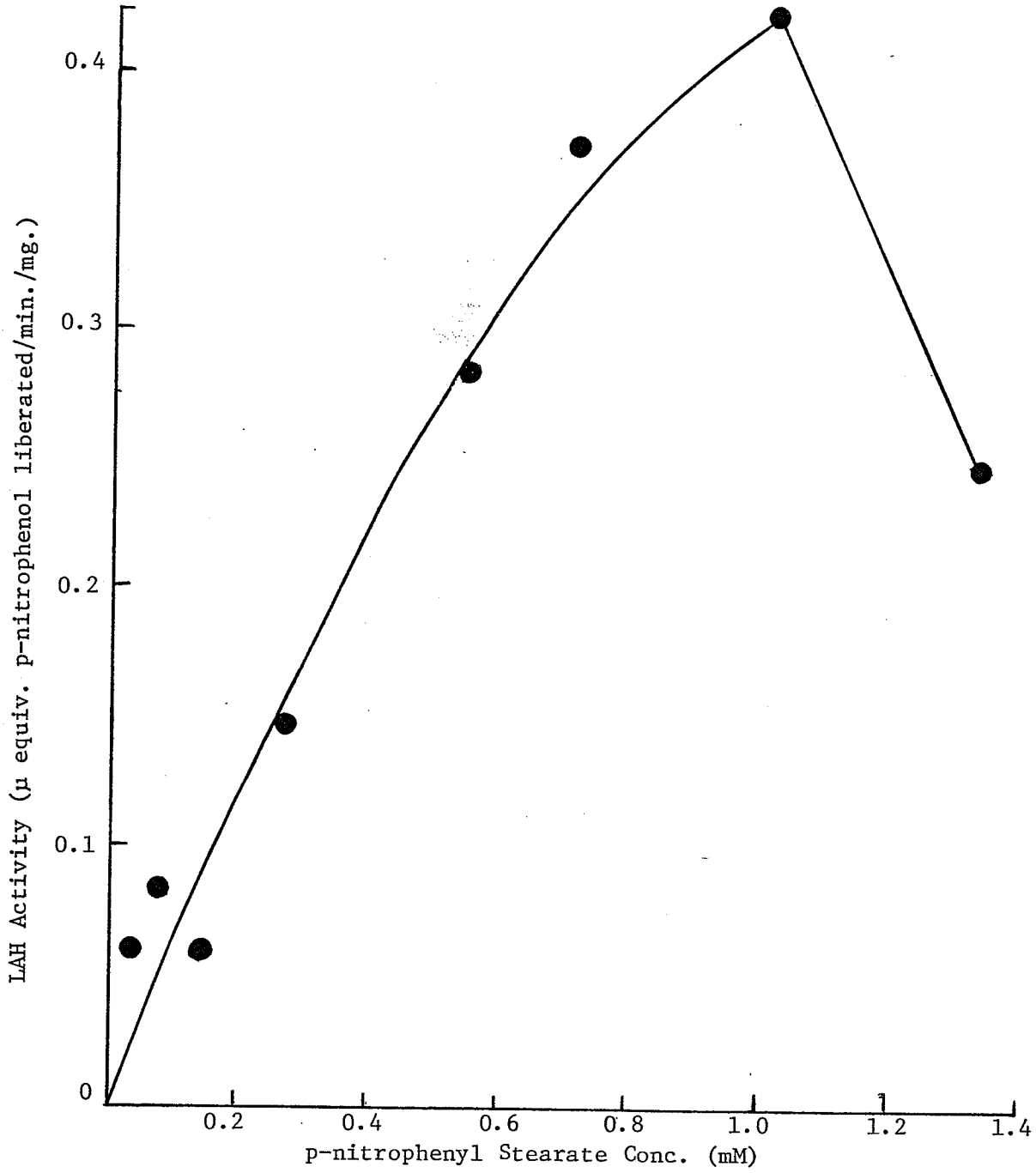


Figure 5. Effect of p-Nitrophenol Stearate Concentration on Fababean Lipolytic Acyl Hydrolase (pH 8.5).

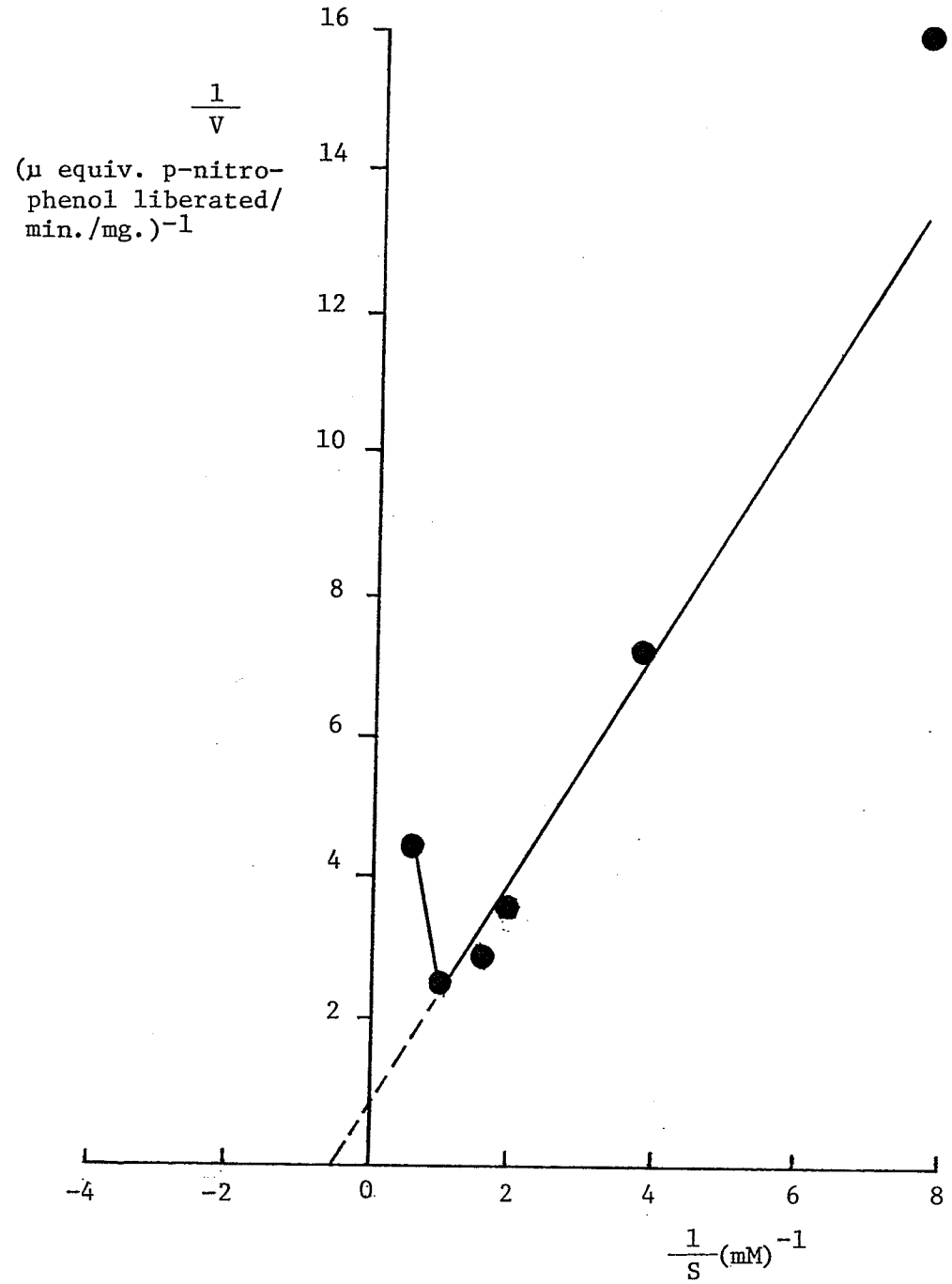


Figure 6. Lineweaver-Burk Plot of the Effect of p-nitrophenyl Stearate Concentration on Fababean Lipolytic Acyl Hydrolase Activity (pH 8.5)

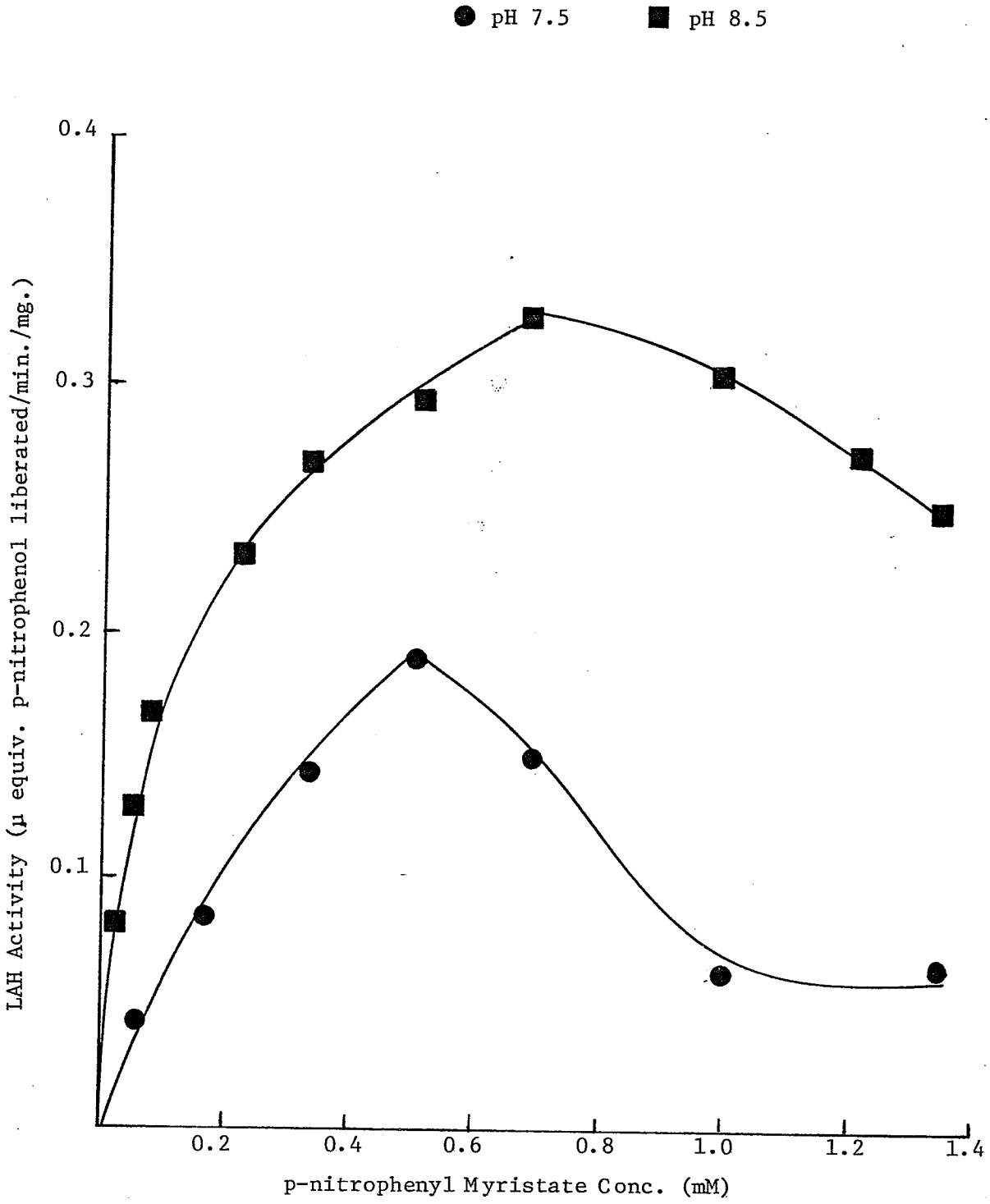


Figure 7. Effect of p-Nitrophenyl Myristate Concentration on Fababean Lipolytic Acyl Hydrolase.

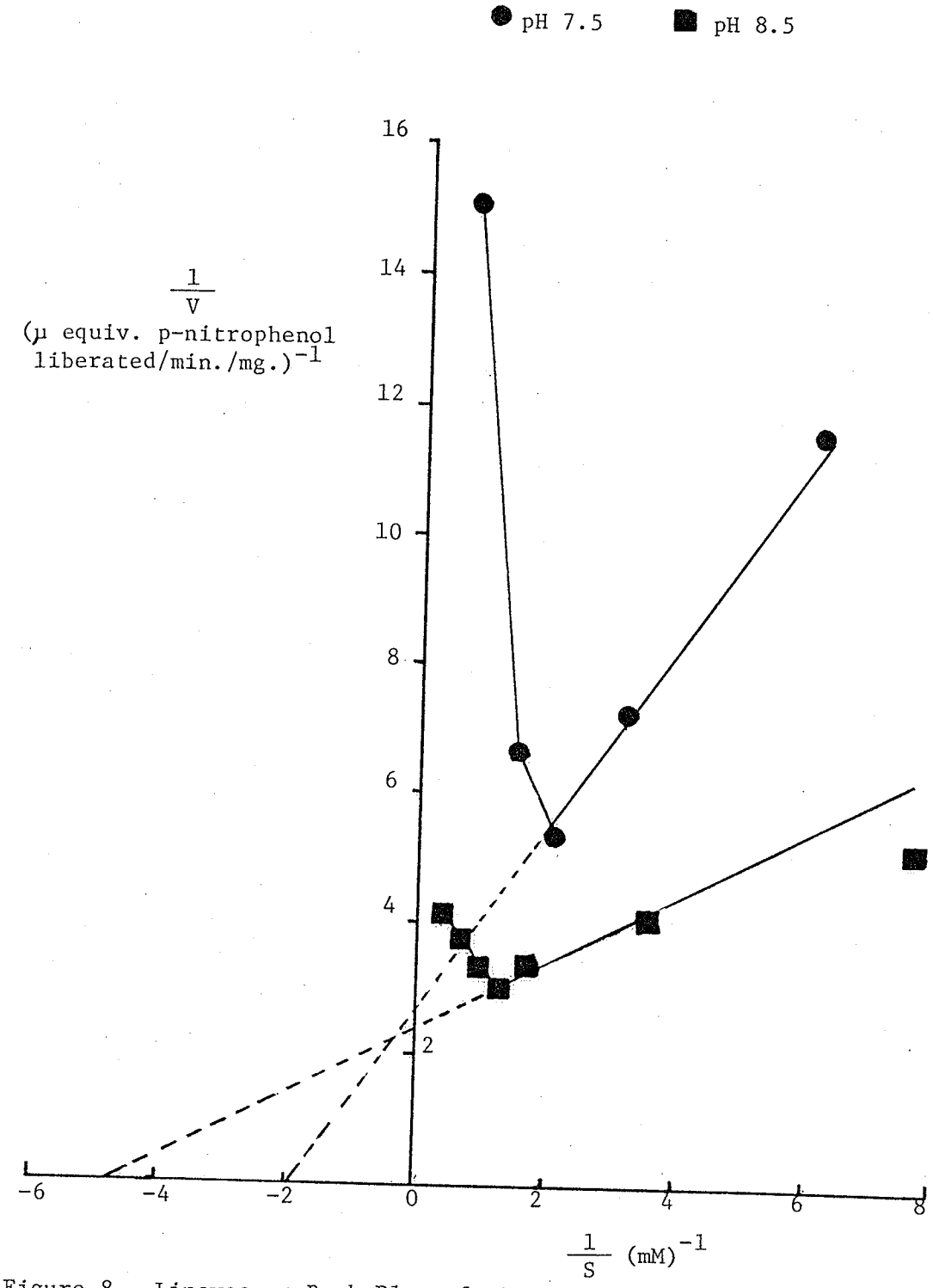


Figure 8. Lineweaver-Burk Plot of the Effect of p-Nitrophenyl Myristate Concentration on Fababean Lipolytic Acyl Hydrolase Activity.

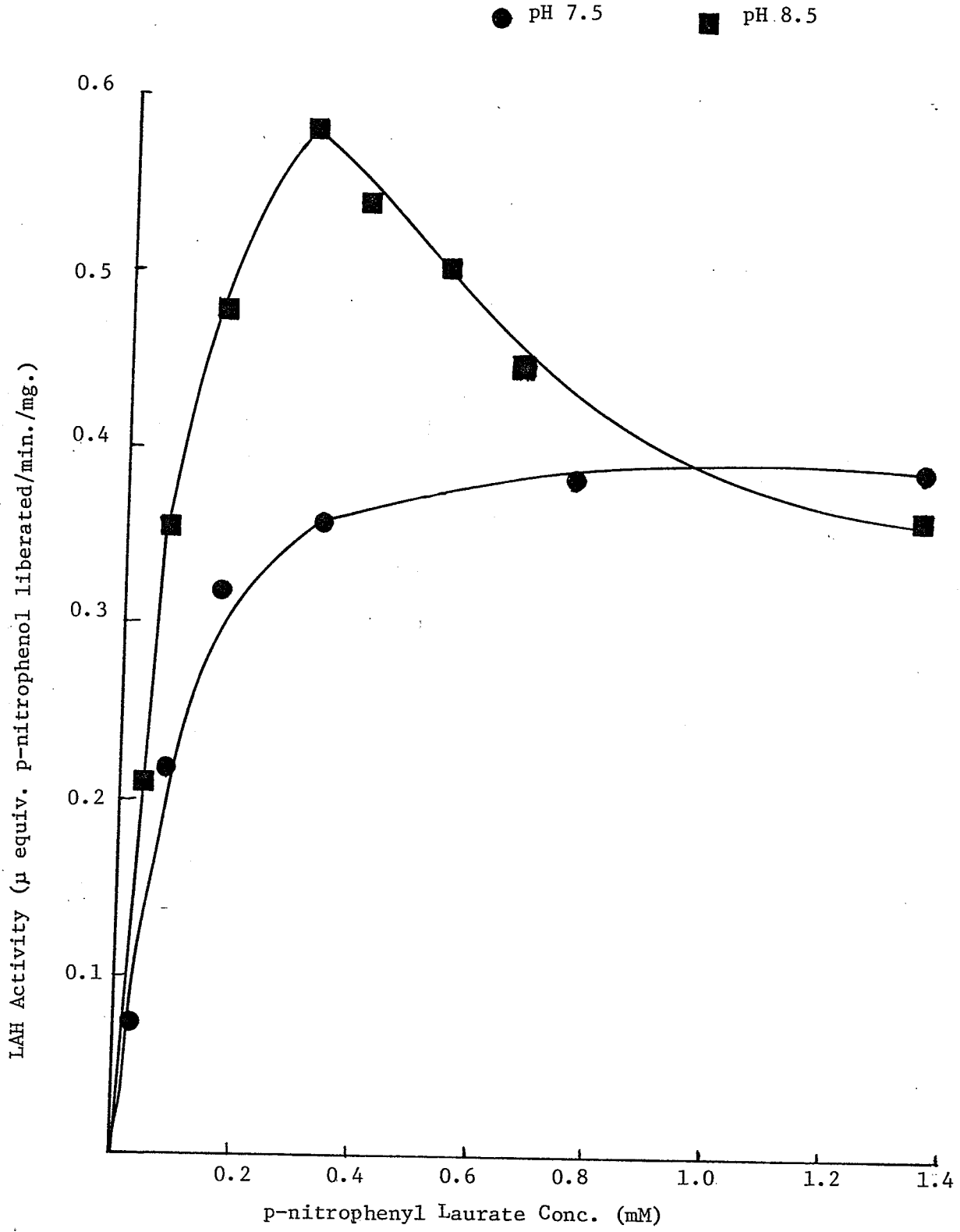


Figure 9. Effect of p-nitrophenyl Laurate Concentration on Fababean Lipolytic Acyl Hydrolase.

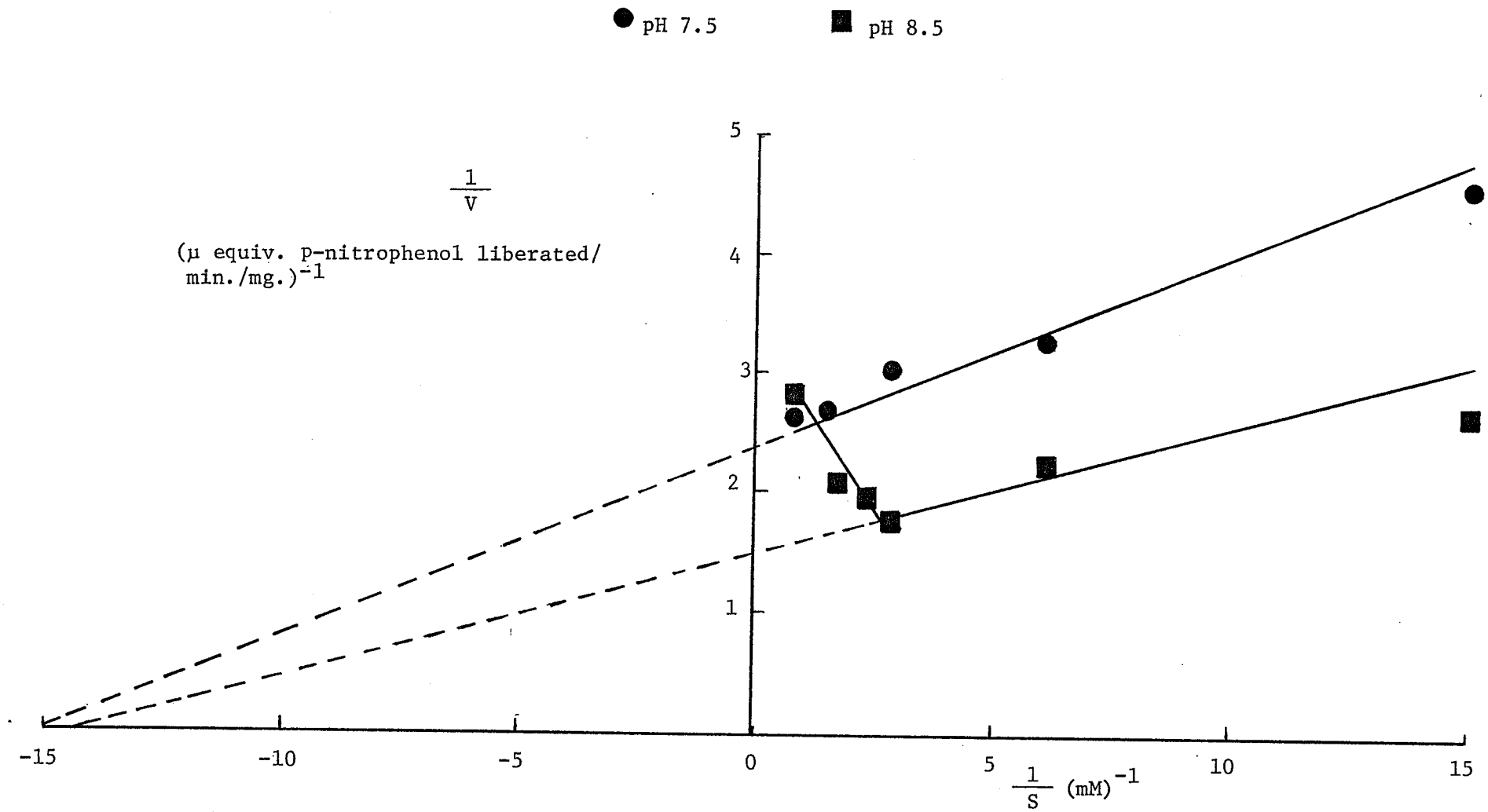


Figure 10. Lineweaver-Burk Plot of the Effect of p-nitrophenyl Laurate on Fababean Lipolytic Acyl Hydrolase Activity.

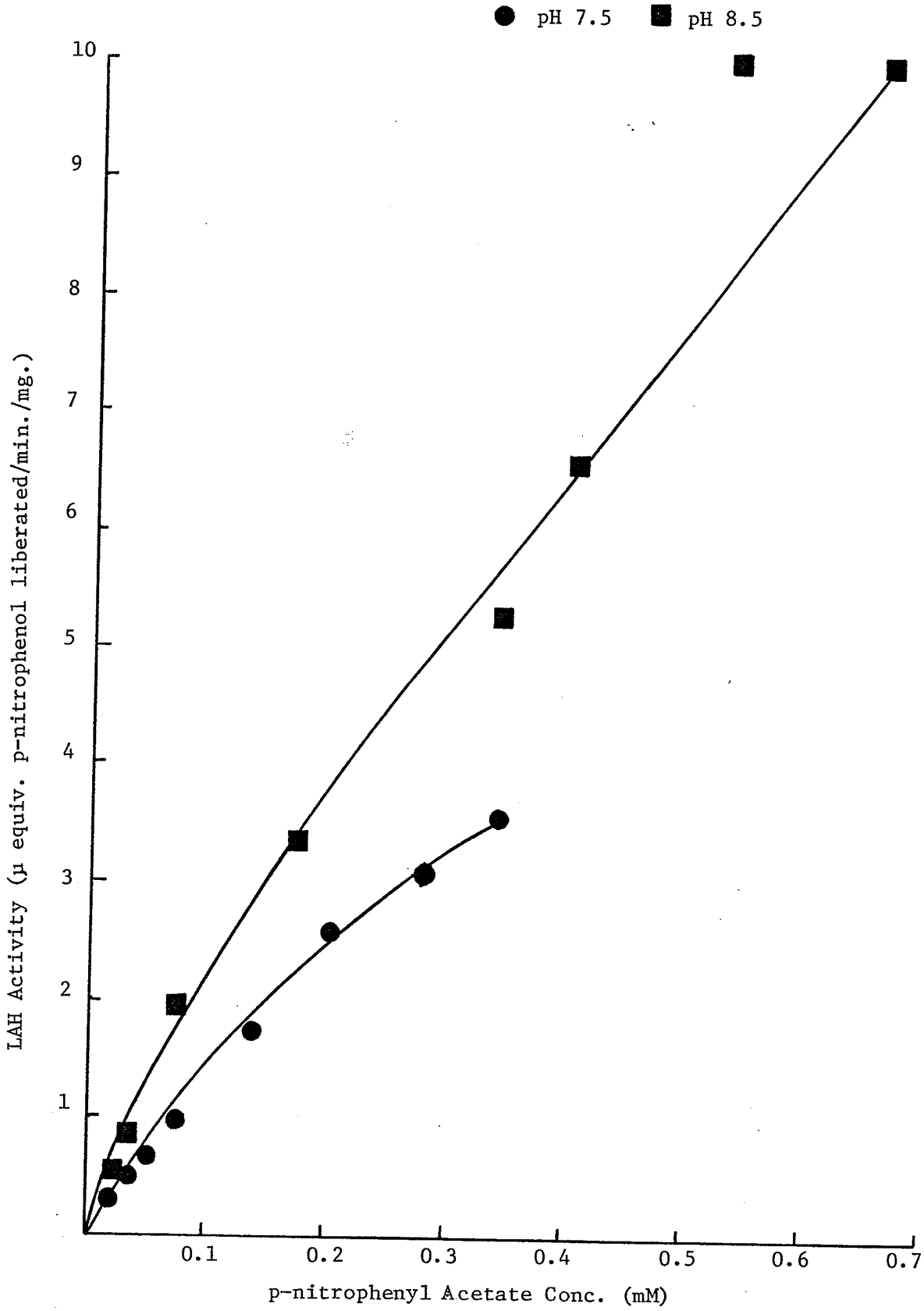


Figure 11. Effect of p-nitrophenyl Acetate Concentration on Fababean Lipolytic Acyl Hydrolase.

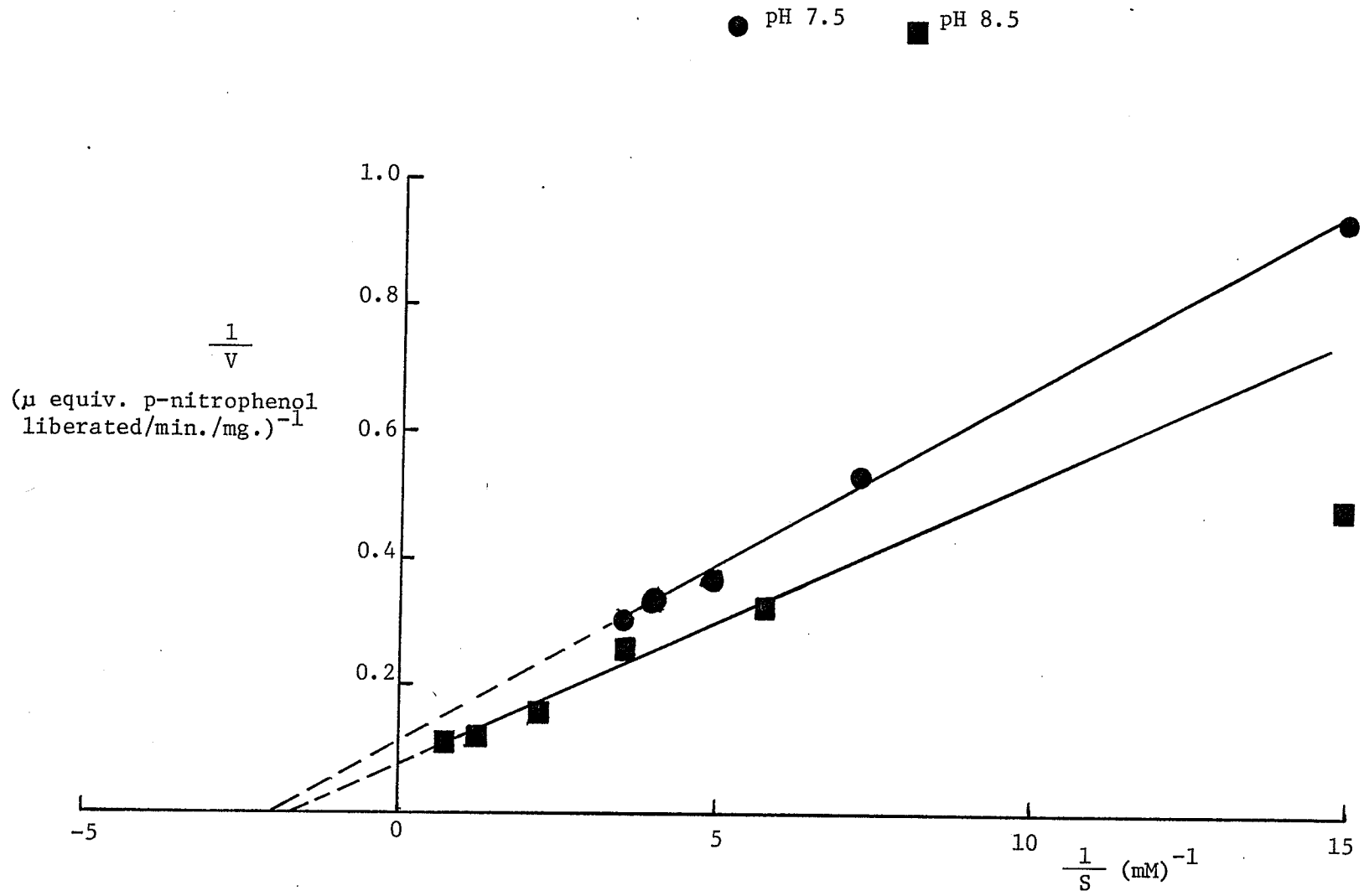


Figure 12. Lineweaver-Burk Plot of the Effect of p-nitrophenyl Acetate Concentration on Fababean Lipolytic Acyl Hydrolase Activity.

Table 1. Calculated Values of Vmax and Km for Substrates Assayed with Fababean Lipolytic Acyl Hydrolase.

Substrate	pH	p-nitrophenyl Ester Hydrolase Assay		Decrease in Acyl Ester Content	
		Vmax[1]	Km[2]	Vmax	Km[3]
p-nitrophenyl acetate	7.5	10.00	0.59	-	-
	8.5	16.67	1.43	-	-
p-nitrophenyl laurate	7.5	0.418	0.07	-	-
	8.5	0.704	0.67	-	-
p-nitrophenyl myristate	7.5	0.400	0.50	-	-
	8.5	0.435	0.22	-	-
p-nitrophenyl palmitate	7.5	0.308	0.63	-	-
	8.5	0.333	0.29	-	-
p-nitrophenyl stearate	7.5	-	-	-	-
	8.5	1.111	1.82	-	-
phosphatidyl-choline[4]	5.6	-	-	0.127	0.36
	7.5	-	-	0.105	1.43
1,2 dilinolein[5]	5.6	-	-	0.250	1.43

[1] Units = μ equiv. p-nitrophenol liberated/min./mg.

[2] Units = mM

[3] Units = μ equiv. ester/ml.

[4] Vmax Units = μ equiv. ester hydrolysed / 10 min./mg.

[5] Vmax Units = μ equiv. ester hydrolysed / 30 min./mg.

5.2.2 Phospholipid Substrates

Various phospholipid substrates were assayed with fababean lipolytic acyl hydrolase, and the initial velocities in the presence of various concentrations of each substrate were determined and plotted against their respective substrate concentrations. The effect of substrate concentration on lipolytic acyl hydrolase activity is presented for phosphatidylcholine, 1,2 dilinolein and phosphatidic acid in Figures 13, 16 and 18 respectively, and is shown as a rectangular hyperbole in each case. There is no indication of the substrate-inhibition effect as demonstrated with higher concentrations of the p-nitrophenyl esters (a slight decrease in phospholipase activity is indicated for the hydrolysis of the two highest concentrations of phosphatidylcholine at pH 7.5).

Lineweaver-Burk plots of the data for the hydrolysis of phosphatidylcholine at both pH 5.6 and 7.5 (Figures 14 and 15) gave straight lines which could be extrapolated to determine Km values of 0.18 mM (pH 5.6) and 0.72 mM (pH 7.5).

Difficulty was encountered in attempts to obtain a stable emulsion in the case of 1,2 dilinolein and phosphatidic acid (Figures 16 and 18). All of the weighed sample would not go into solution, and the exact amount that did could not be determined until after the assay was completed, the result being that little control was held over the amount of substrate added to each reaction mixture. Due to the great cost of these two substrates and the resulting small amount of substrate available, few trials could be carried out. Regardless of this, a curve was obtained when fababean lipolytic acyl hydrolase was assayed with 1,2 dilinolein (Figure 16). The corresponding Lineweaver-Burk plot (Figure 17) gave

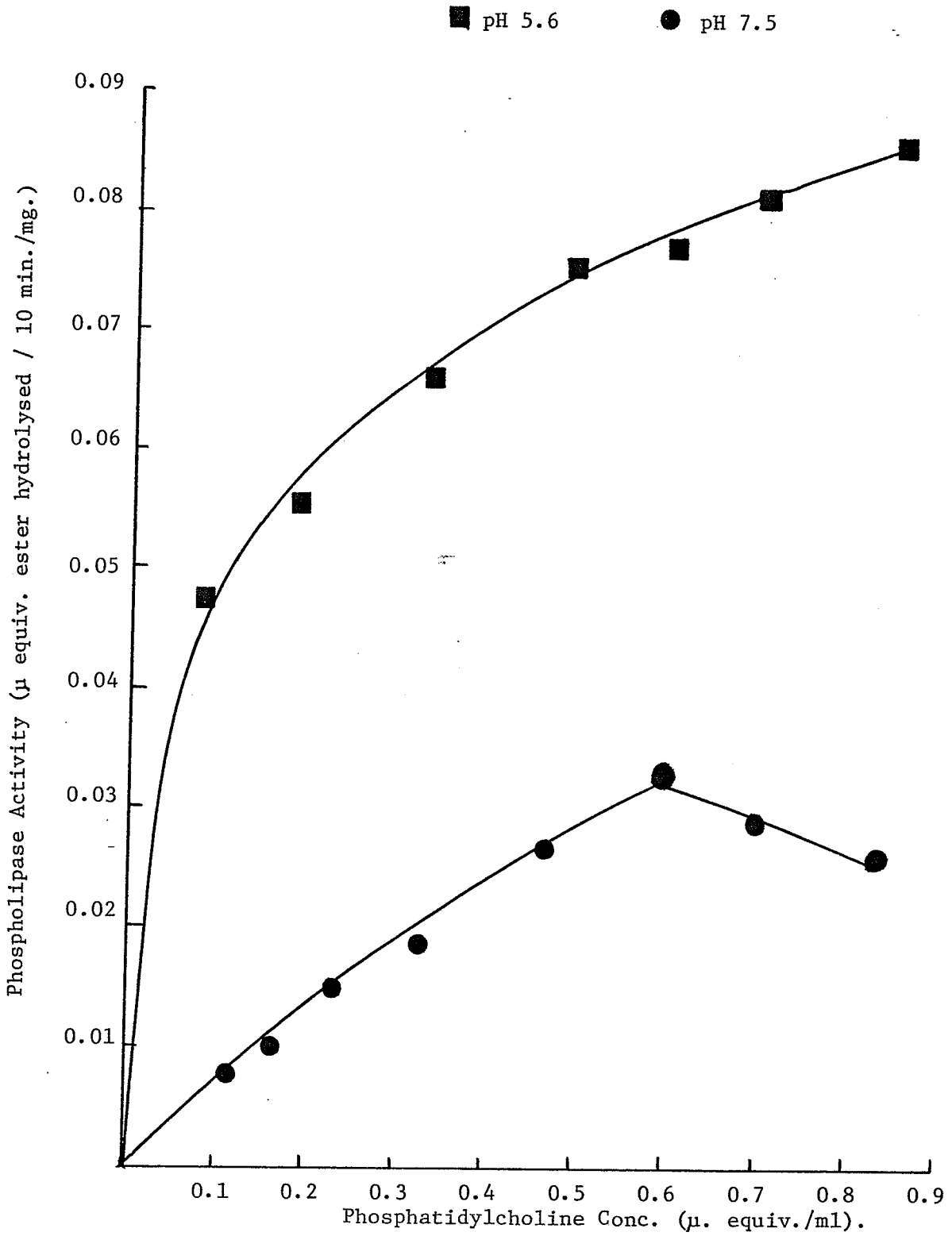


Figure 13. Effect of Phosphatidylcholine Concentration on Fababean Phospholipase Activity.

Figure 14. Lineweaver-Burk Plot of the Effect of Phosphatidylcholine Concentration on Fababean Phospholipase (pH 5.6)

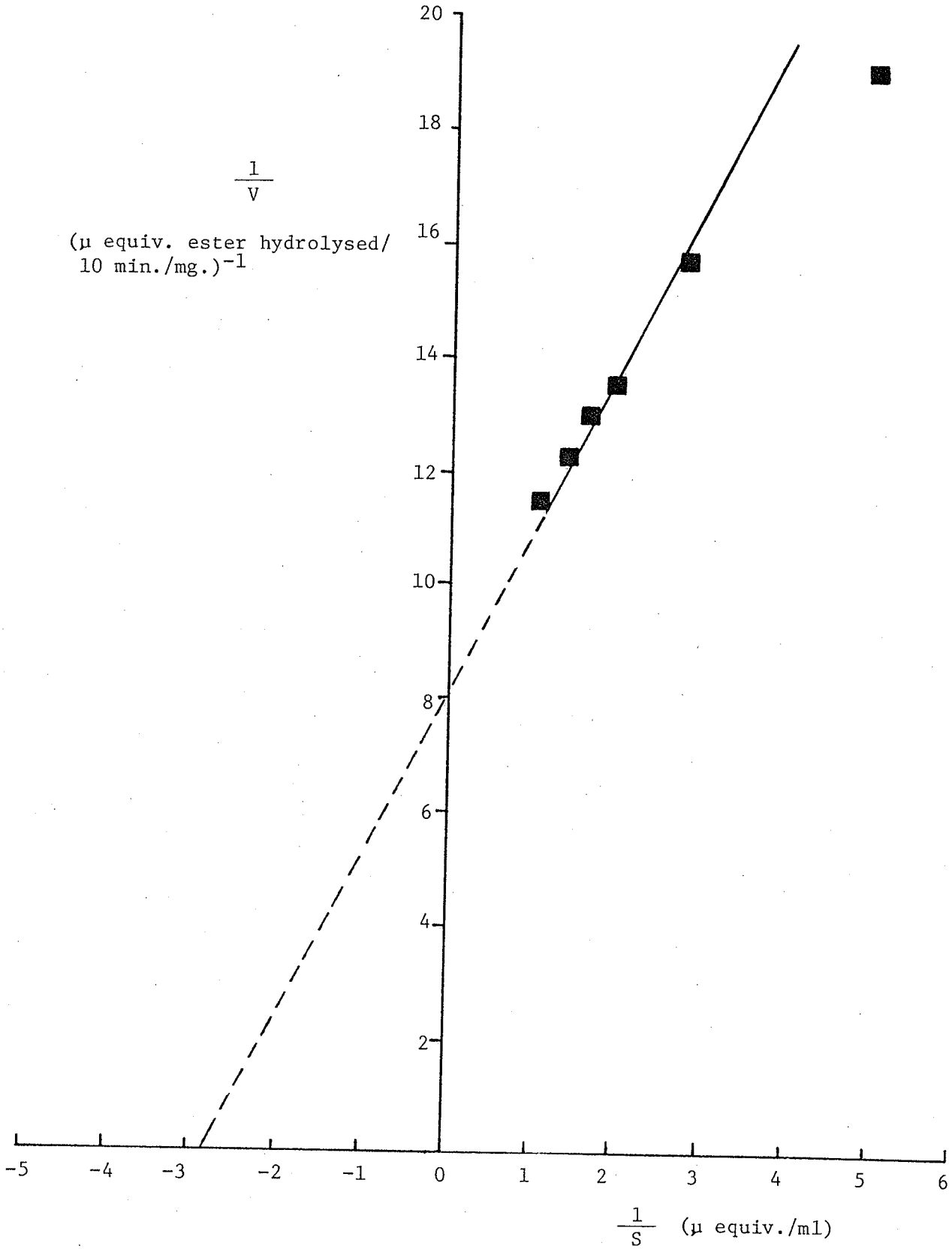
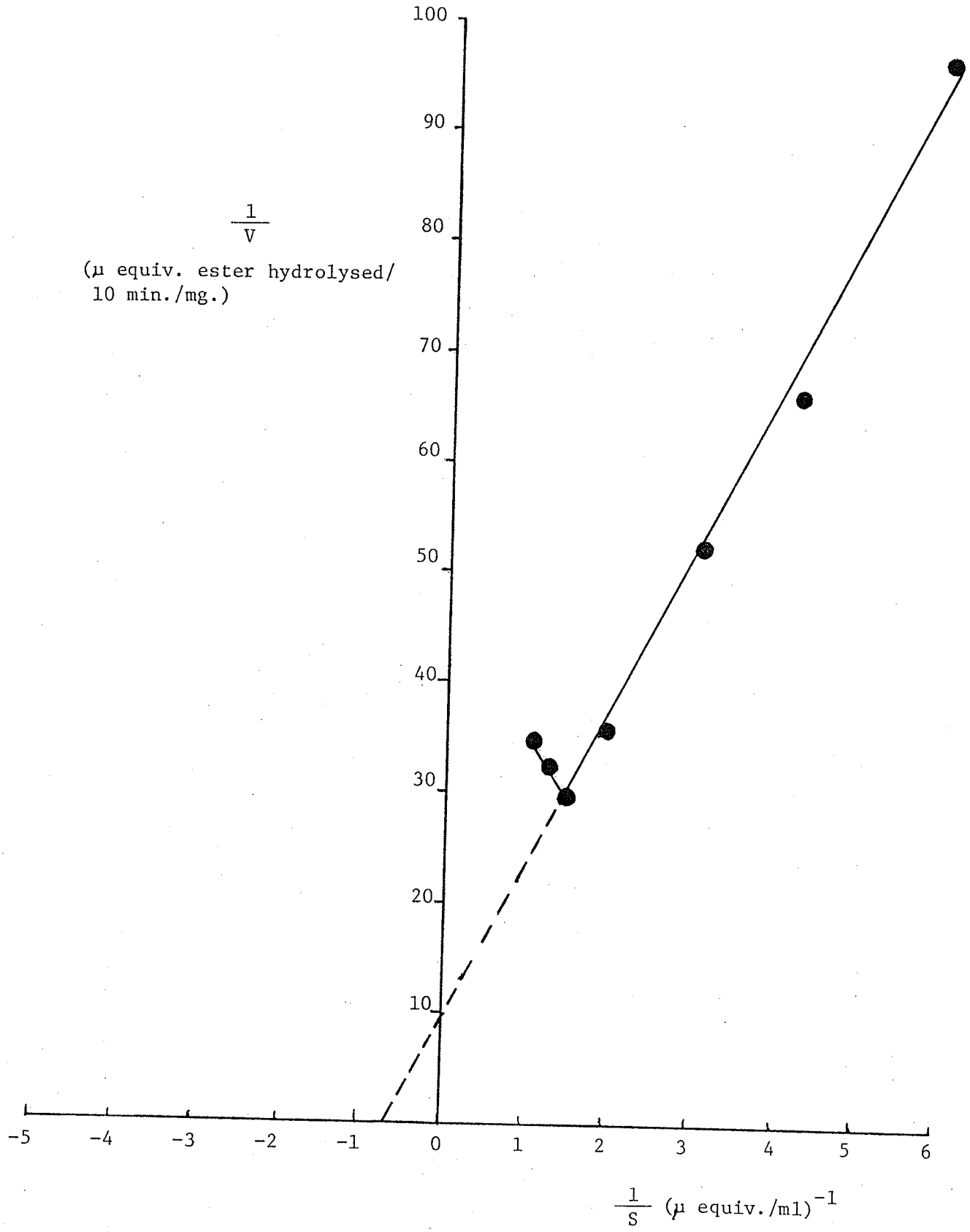


Figure 15. Lineweaver-Burk Plot of the Effect of Phosphatidylcholine Concentration on Fababean Phospholipase (pH 7.5).



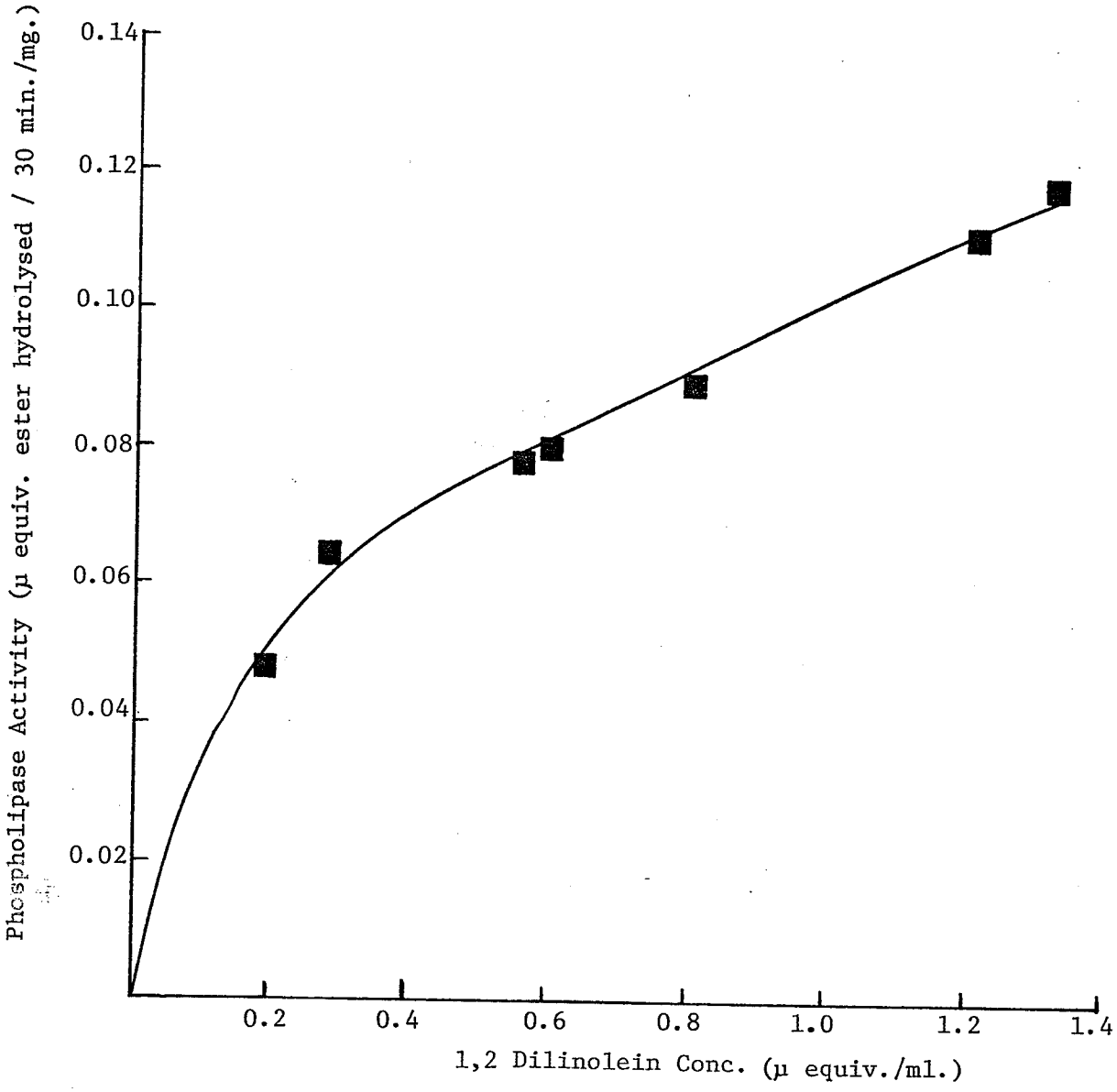


Figure 16. Effect of 1,2 Dilinolein Concentration on Fababean Phospholipase Activity (pH 5.6).

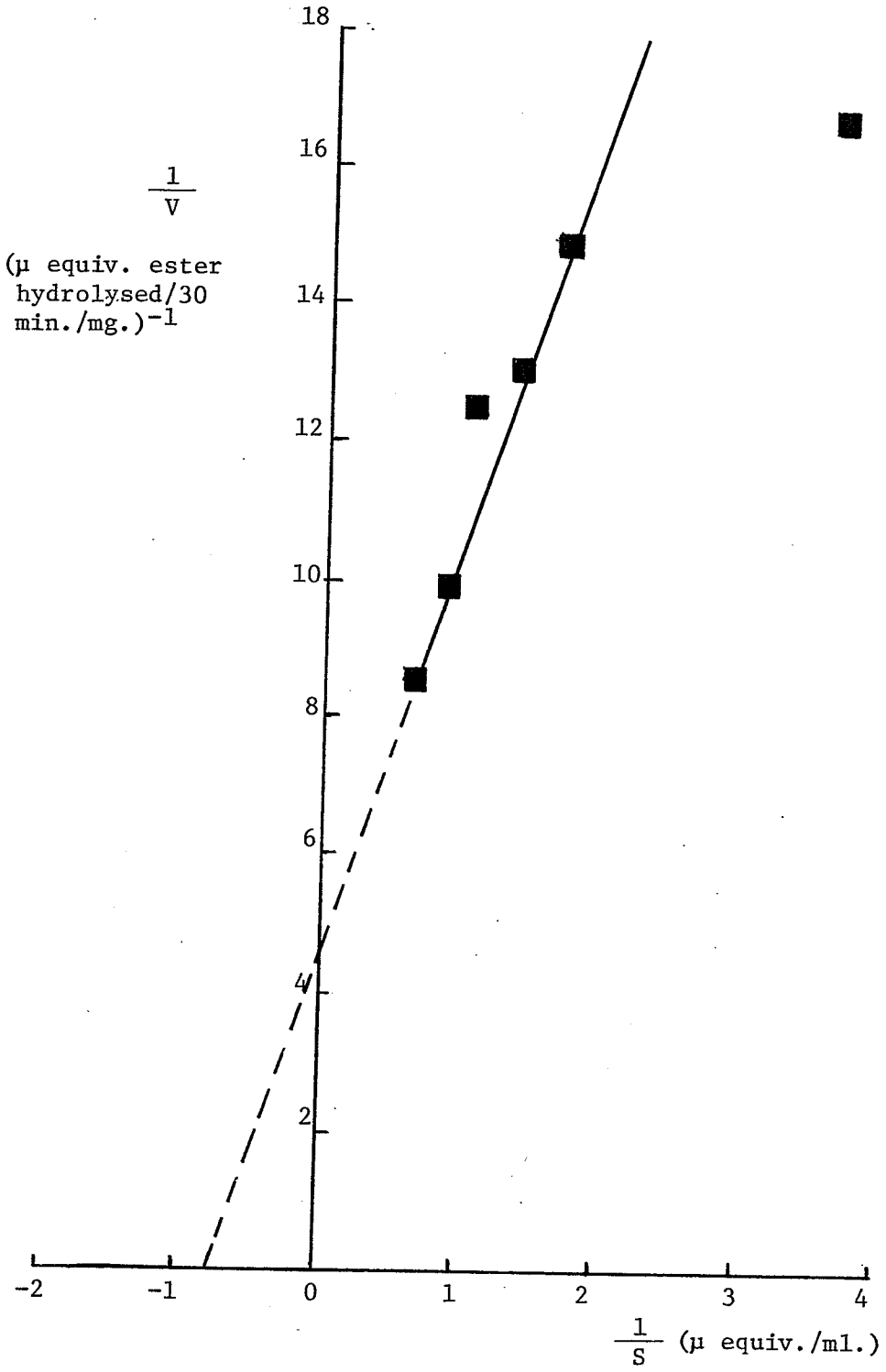


Figure 17. Lineweaver-Burk Plot of the Effect of 1,2 Dilinolein Concentration on Fababean Phospholipase (pH 5.6).

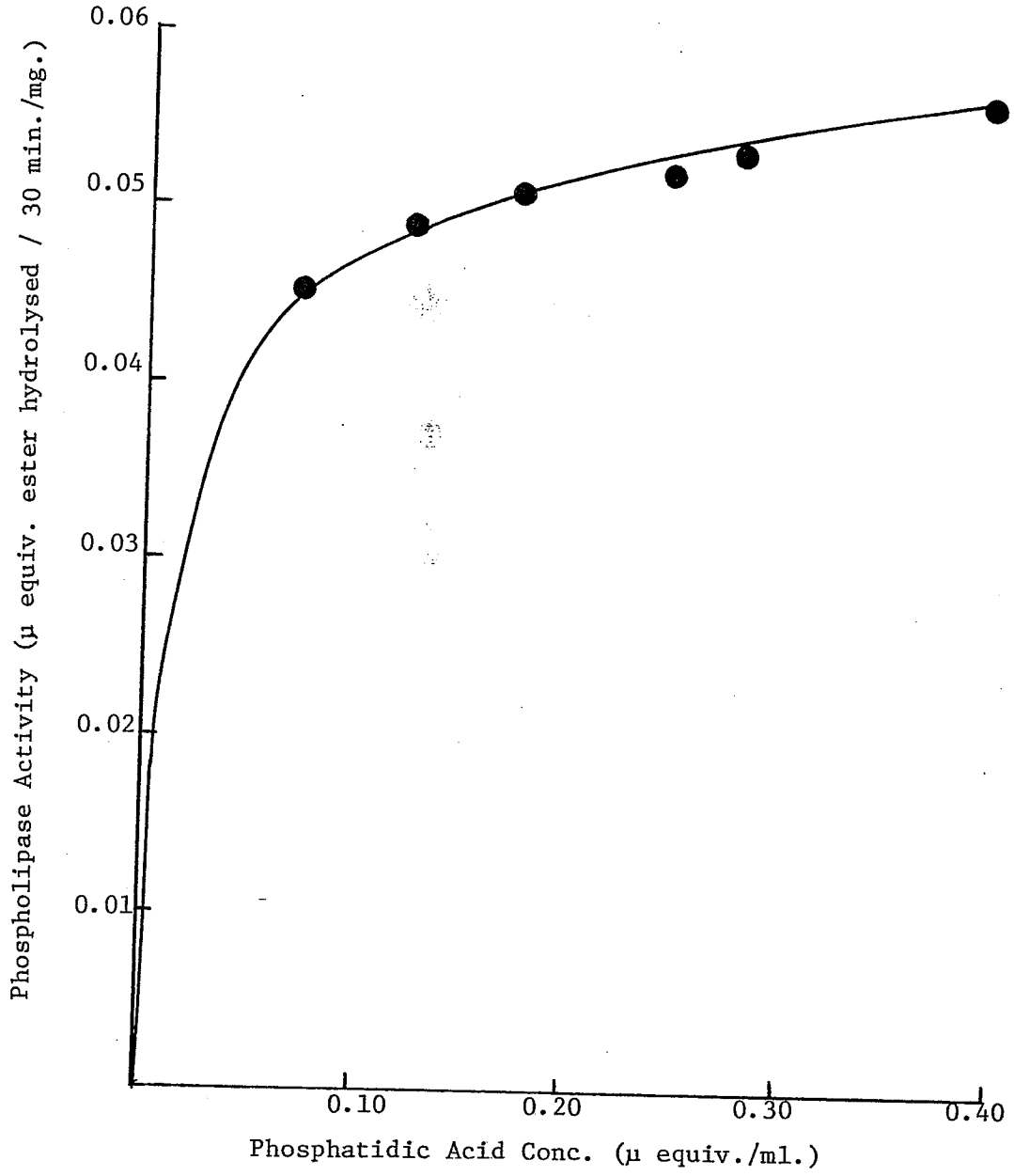


Figure 18. Effect of Phosphatidic Acid Concentration on Fababean Phospholipase Activity (pH 5.6).

a straight line and from the intercept on the abscissa the apparent Km value is 1.43 microequivalents of ester/ml. Proportionately less of the phosphatidic acid substrate went into solution and as a result the assay was run over a very narrow range of concentration (0.0 - 0.4 vs. 0.0 - 1.4 microequivalents of ester/ml.). Because of the narrow range of substrate concentration assayed, insufficient data were available for a valid Lineweaver-Burk plot of phosphatidic acid concentration vs. enzyme activity. Initial trials indicated that little enzyme activity had occurred at the end of the standard 10 minute incubation period, due to the problems in obtaining a stable emulsion and the correspondingly less substrate available for reaction. In order to compensate for this, the incubation period was increased to 30 minutes for assays with 1,2 dilinolein and phosphatidic acid.

In order to determine if the above results obtained with phospholipid substrates were ascribable to fababean lipolytic acyl hydrolase, or to a lipase (Dundas et al., 1978) fraction of the crude fababean extract, tests were run using a commercial preparation of lipase. Crude hog pancreatic lipase (0-12 mg./ml.) was assayed with phosphatidylcholine using the standard phospholipase assay both at pH 5.6, the pH optimum for hydrolysis of phosphatidylcholine with fababean lipolytic acyl hydrolase, and at pH 7.4, the pH optimum of the particular preparation of lipase. Figure 19 demonstrates only residual activity at both pH's of incubation, indicating that deacylation of phospholipid substrates occurs through lipolytic acyl hydrolase activity which in this case may be distinct from lipase which attacks triglycerides.

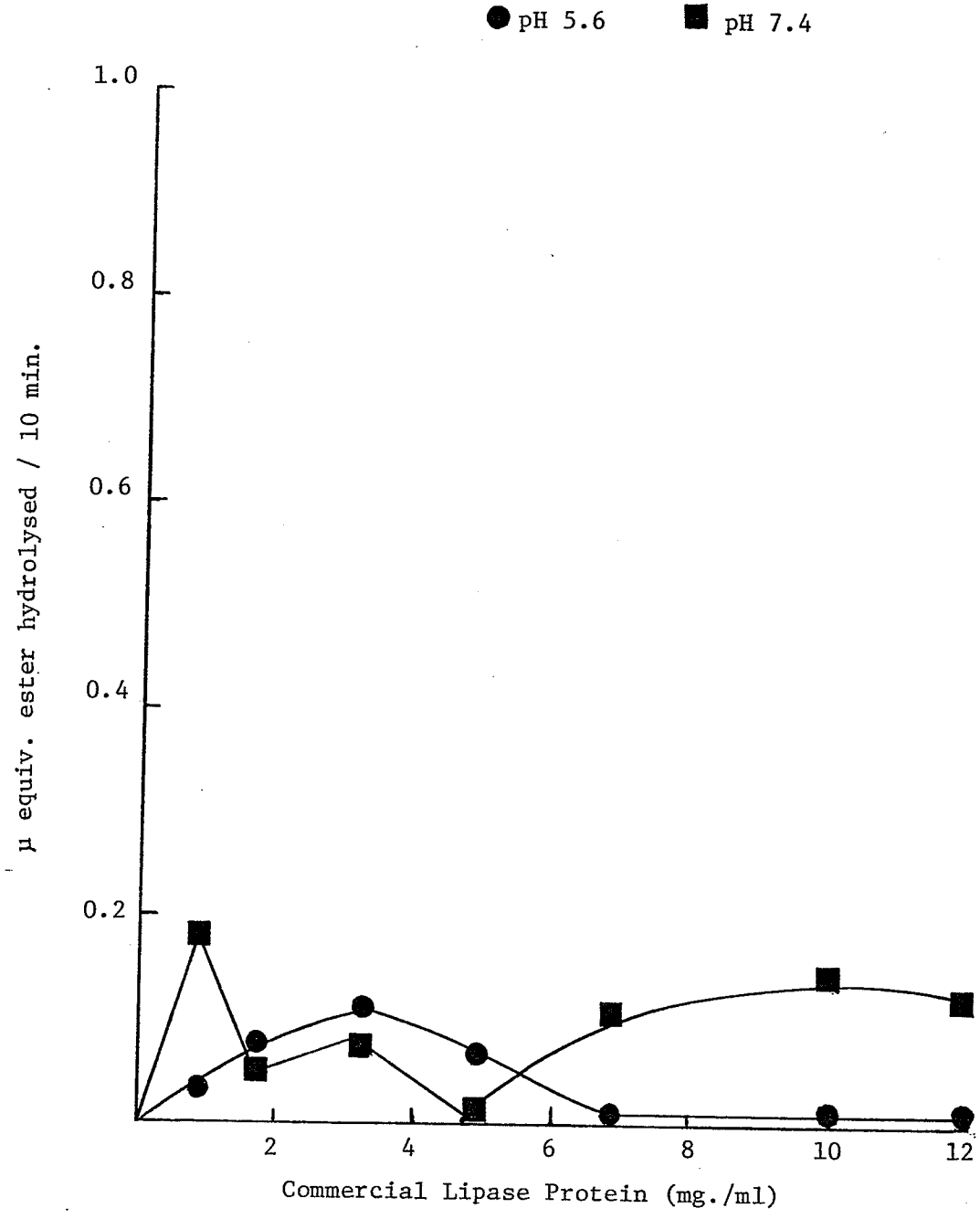


Figure 19. Effect of Lipase Enzyme Concentration on the Deacylation of Phosphatidylcholine.

In order to test the specificity of the spectrophotometric determination of ester groups used in the standard assay procedure (Snyder and Stephens, 1959; Renkonen, 1961), glycerophosphate, which contains only phosphoric ester linkages, was tested as a substrate. A negative result was obtained as the purple color which normally develops to indicate the presence of acyl ester linkages, did not develop. This indicates that the decrease in ester content of the various phospholipids assayed with fababean lipolytic acyl hydrolase is due to deacylation at positions C_1 and/or C_2 and not due to phospholipase C- or D-type hydrolysis which would also reduce the ester content of the phospholipid substrates.

5.3 pH Profile

The influence of pH on the activity of fababean lipolytic acyl hydrolase is presented in Figures 20 and 21. The pH profile in Figure 20 shows enzyme activity within a pH range of 6.0 - 9.0 with the optimum at pH 8.5 using p-nitrophenyl palmitate as substrate. Activity could not be measured above pH 9.0 due to instantaneous hydrolysis of the p-nitrophenyl substrate in the absence of enzyme. This effect was present, although to a much lesser degree, below pH 6.5.

Figure 21 demonstrates the effect of pH on the hydrolysis of phosphatidylcholine by fababean lipolytic acyl hydrolase. Activity was determined over the pH range of 4.5 to 8.5. Non-enzymic hydrolysis of the substrate occurred at pH values beyond this range. The pH optimum is 5.6 with a possible secondary optimum at pH 7.5.

5.4 Effect of Temperature

The effect of temperature on fababean lipolytic acyl hydrolase

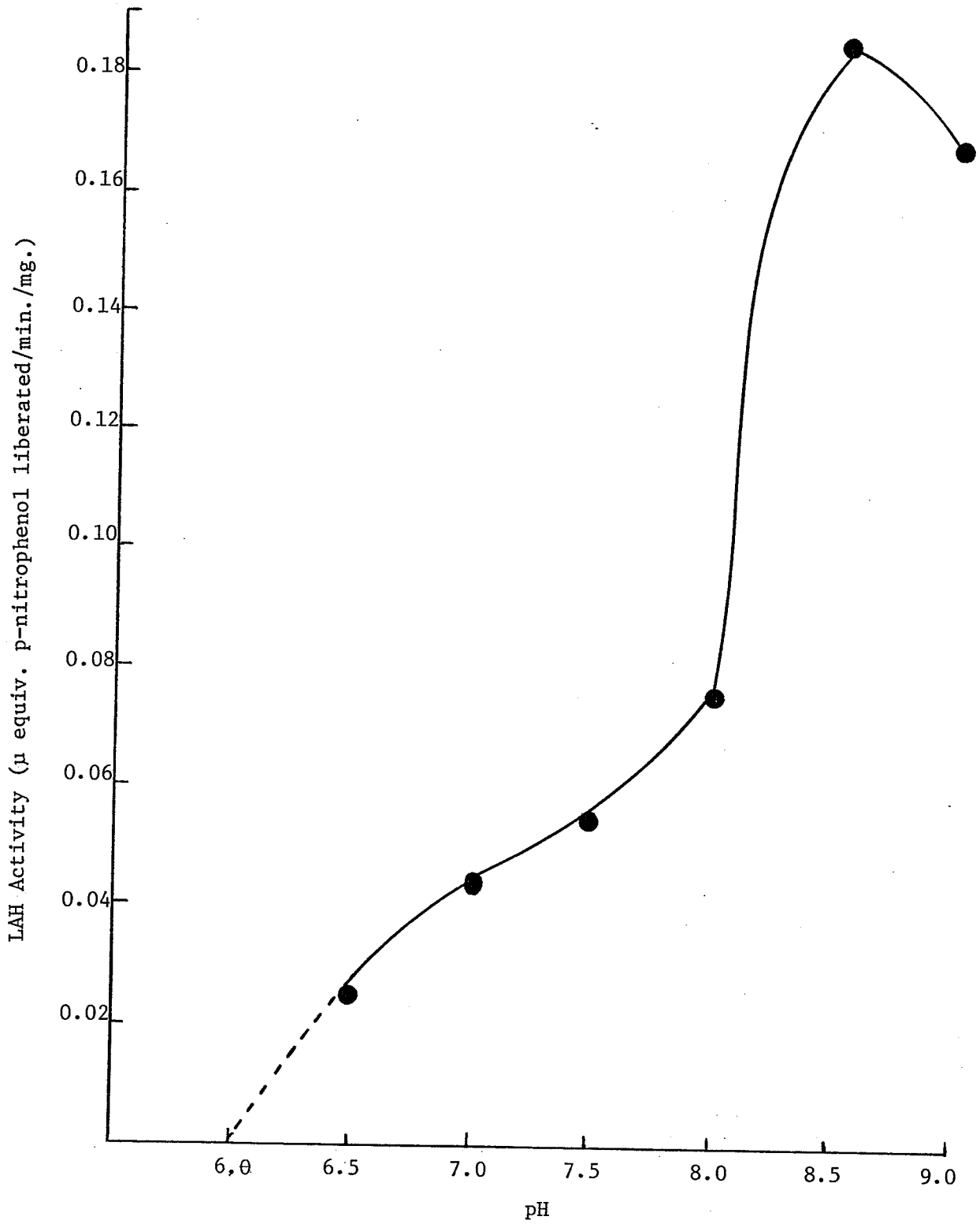


Figure 20. Effect of pH on Fababean p-nitrophenyl Ester Hydrolase Activity.

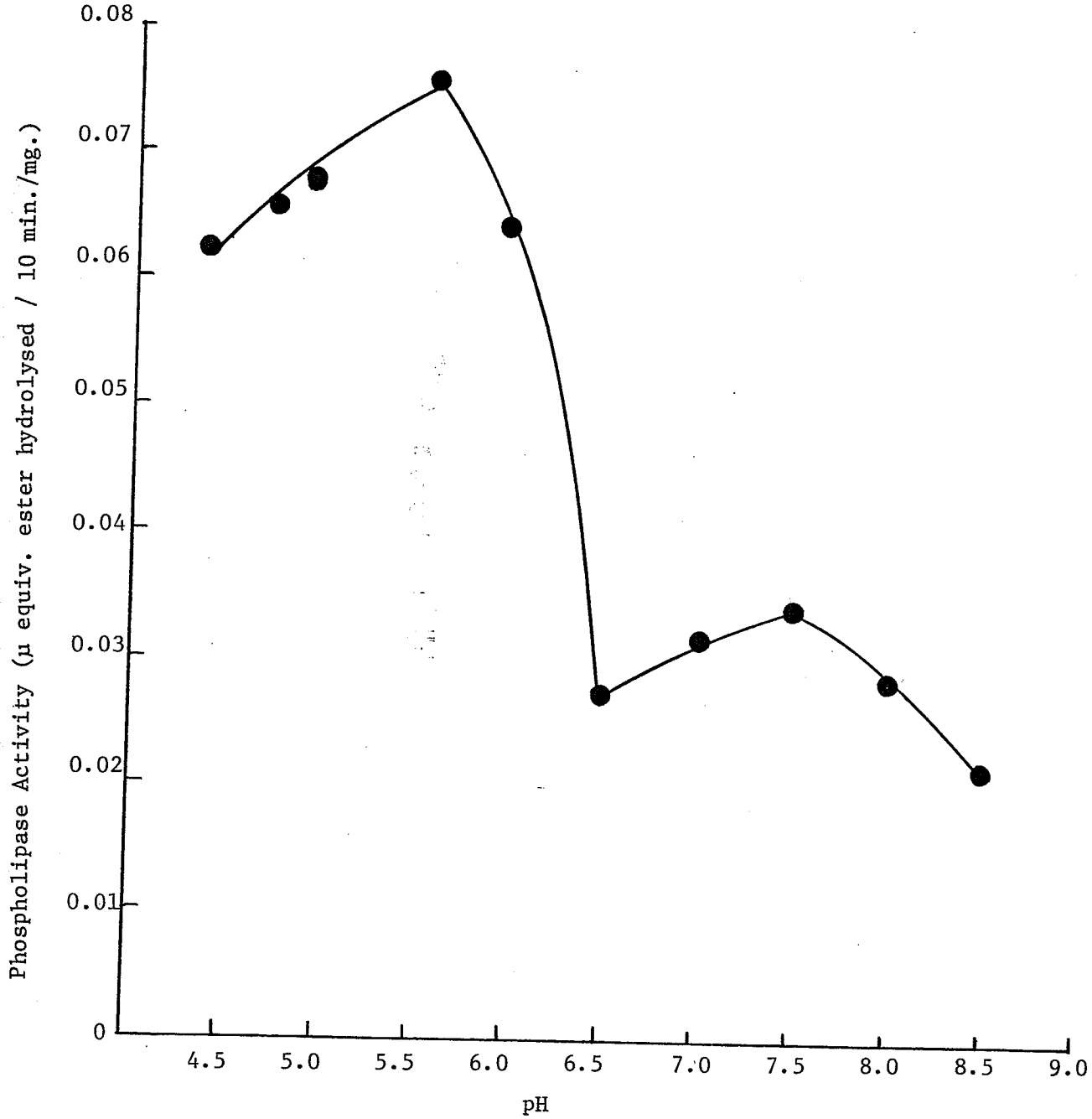


Figure 21. Effect of pH on Fababean Phospholipase Activity.

was studied, and the results are presented in Figure 22. Both the water bath temperature and the actual temperature reached inside the cuvette at the end of the first minute of assay were monitored, the latter figure being the temperature of assay. Activity was monitored over a water bath temperature range of 25° to 55°C; at temperatures above 55°C, nonenzymic hydrolysis of the p-nitrophenyl palmitate substrate would occur. Figure 22 demonstrates enzyme activity over the temperature range tested, with an optimum temperature of 37°C indicated.

Stability of the enzyme to temperature is shown in Figure 23. Fababean lipolytic acyl hydrolase is stable at 30°C for 10 minutes and will retain 85% of its activity at 37°C for 10 minutes. Incubation at 55°C for 10 minutes results in a 55% reduction in enzyme activity, while exposure for 2 minutes at 75°C completely inhibits enzyme activity. Similar temperature-stability data were reported for a crude lipolytic acyl hydrolase from potato tubers (Galliard, 1971a).

5.5 Effect of Triton X-100 Concentration

The non-ionic detergent Triton X-100 was routinely used in the preparation of all lipid substrates at a concentration of 4 mg./ml. (2 mg./microequivalent of ester in substrate), as this was the reported optimum value (Galliard, 1971a; Dennis, 1973b). The effect of Triton X-100 concentration on fababean lipolytic acyl hydrolase activity was studied with both p-nitrophenyl palmitate and phosphatidyl choline as substrates (Figures 24 and 25 respectively). Hydrolysis of p-nitrophenyl palmitate was optimal at a Triton X-100 concentration of 6 mg./ml, while higher concentrations (8-16 mg./ml.) were found

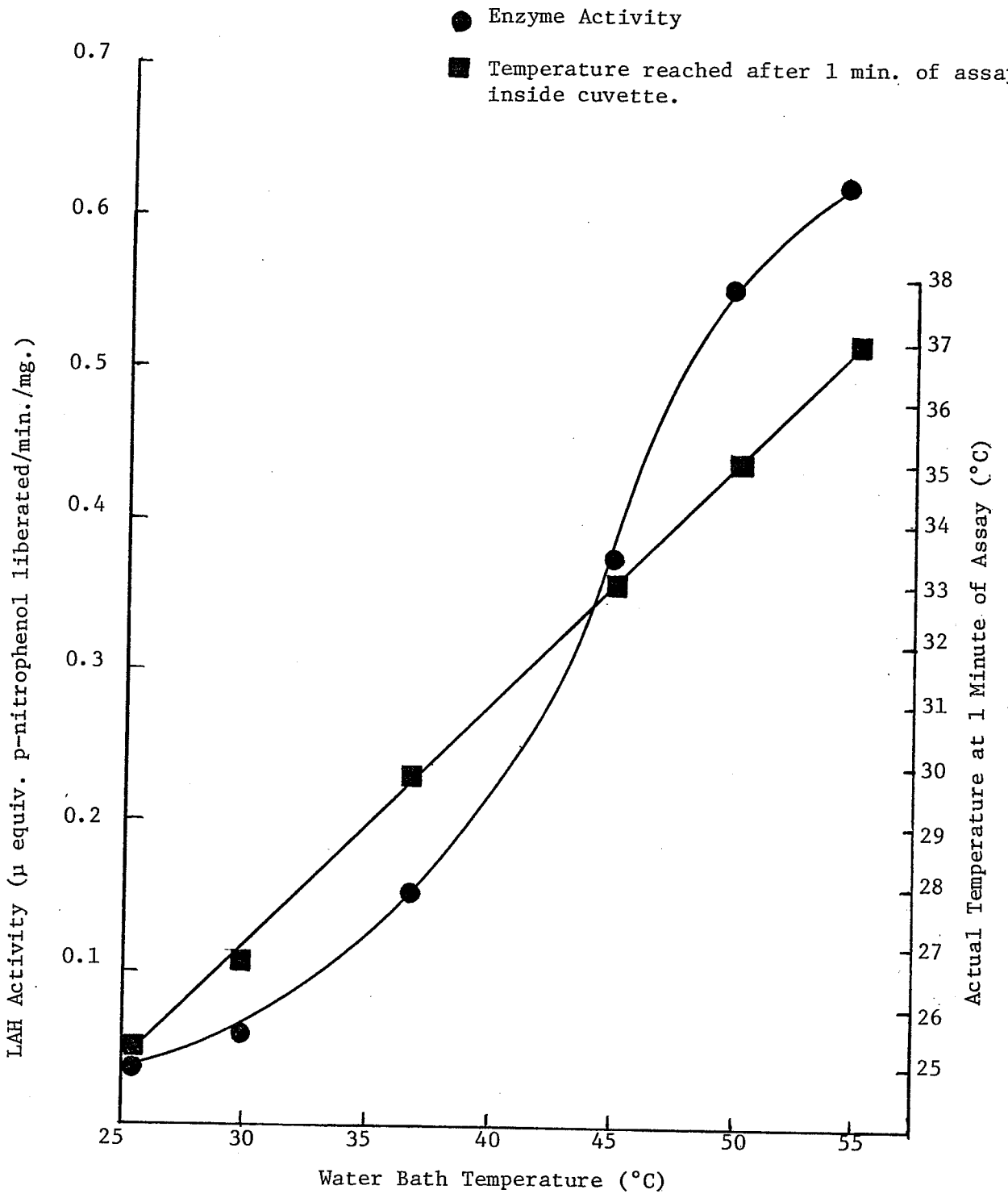


Figure 22. Effect of Temperature (Optimum Temperature) on Fababean Lipolytic Acyl Hydrolase.

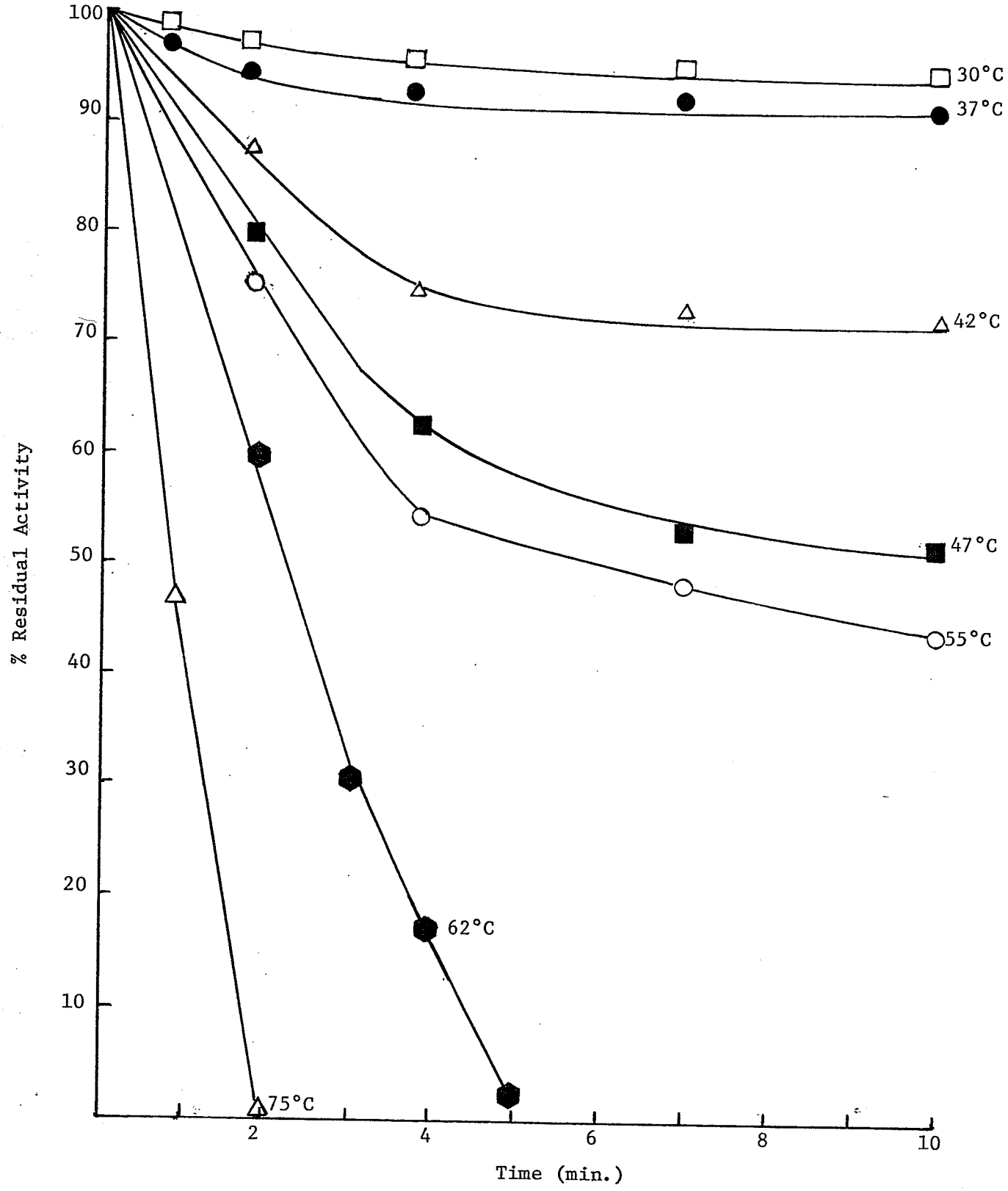


Figure 23. Temperature Stability of Fababean Lipolytic Acyl Hydrolase.

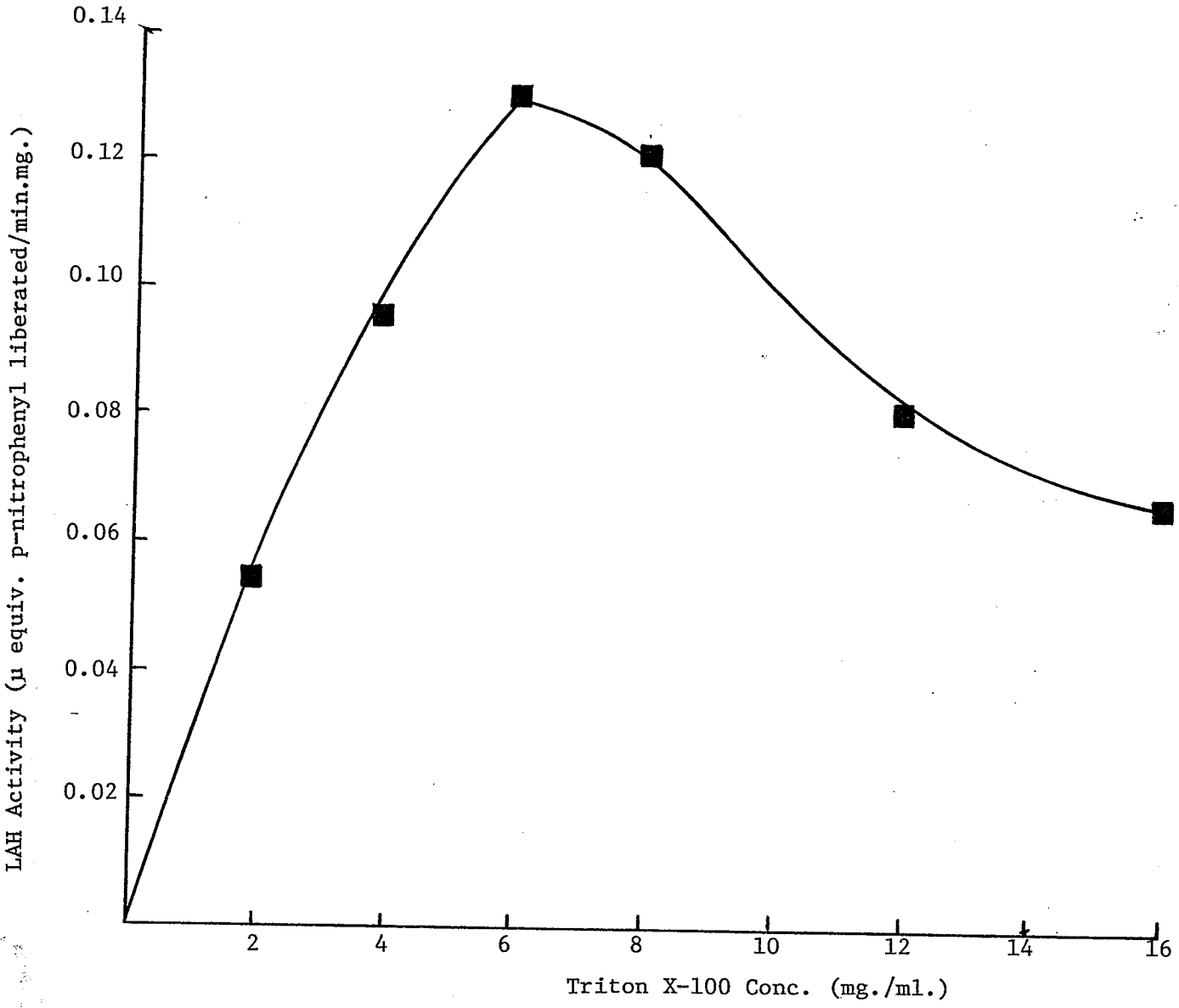


Figure 24. Effect of Triton X-100 Concentration on Fababean Lipolytic Acyl Hydrolase (p-nitrophenyl palmitate substrate).

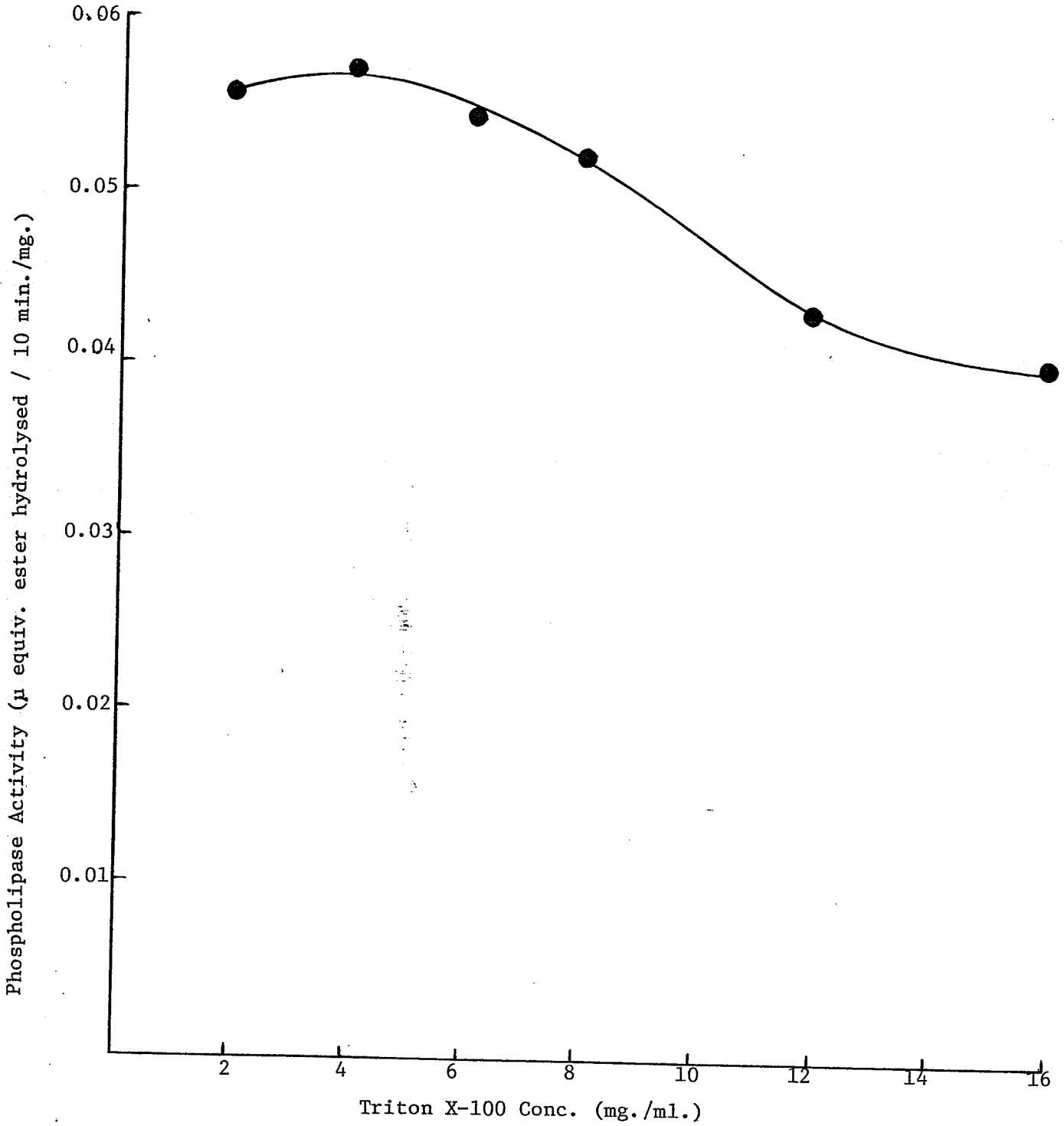


Figure 25. Effect of Triton X-100 Concentration on Fababean Lipolytic Acyl Hydrolase (phosphatidylcholine substrate).

to be inhibitory. Figure 25 demonstrates a broader range of optimal activity (2 - 6 mg./ml. Triton X-100) for the hydrolysis of phosphatidylcholine with a slight peak at 4 mg/ml. Higher concentrations of Triton were found to be inhibitory (8 - 16 mg/ml.).

5.6 Action of Calcium Chloride

The effect of calcium chloride on the activity of fababean lipolytic acyl hydrolase with p-nitrophenyl palmitate is presented in Figure 26. Calcium chloride, a known activator of many deacylating enzymes, stimulates p-nitrophenyl palmitate hydrolysis up to a concentration of 3.0 mM. Activity could not be measured above 3.0 mM $CaCl_2$, because higher concentrations of Ca^{2+} ions appeared to cause the protein to be salted out of solution. Fababean hydrolysis of phosphatidylcholine was not activated by added calcium chloride up to a concentration of 3.0 mM.

5.7 Action of Mercuric Chloride

The activity of fababean lipolytic acyl hydrolase with p-nitrophenyl palmitate was inhibited by mercuric chloride, with 40% of the original activity being inhibited by 1.0 mM mercuric chloride (Figure 27). Activity was completely inhibited with a 5.0 mM mercuric chloride concentration. Phosphatidylcholine was also assayed in the presence of mercuric chloride. Figure 29 demonstrates that phospholipase activity was inhibited to a much lesser extent than was p-nitrophenyl palmitate hydrolysis. 2% of activity was inhibited by 1.0 mM $HgCl_2$, while only 6% of activity was lost with 5.0 mM $HgCl_2$.

The mode of inhibition exhibited by mercuric ions on fababean lipolytic acyl hydrolase was determined by the use of a double reciprocal

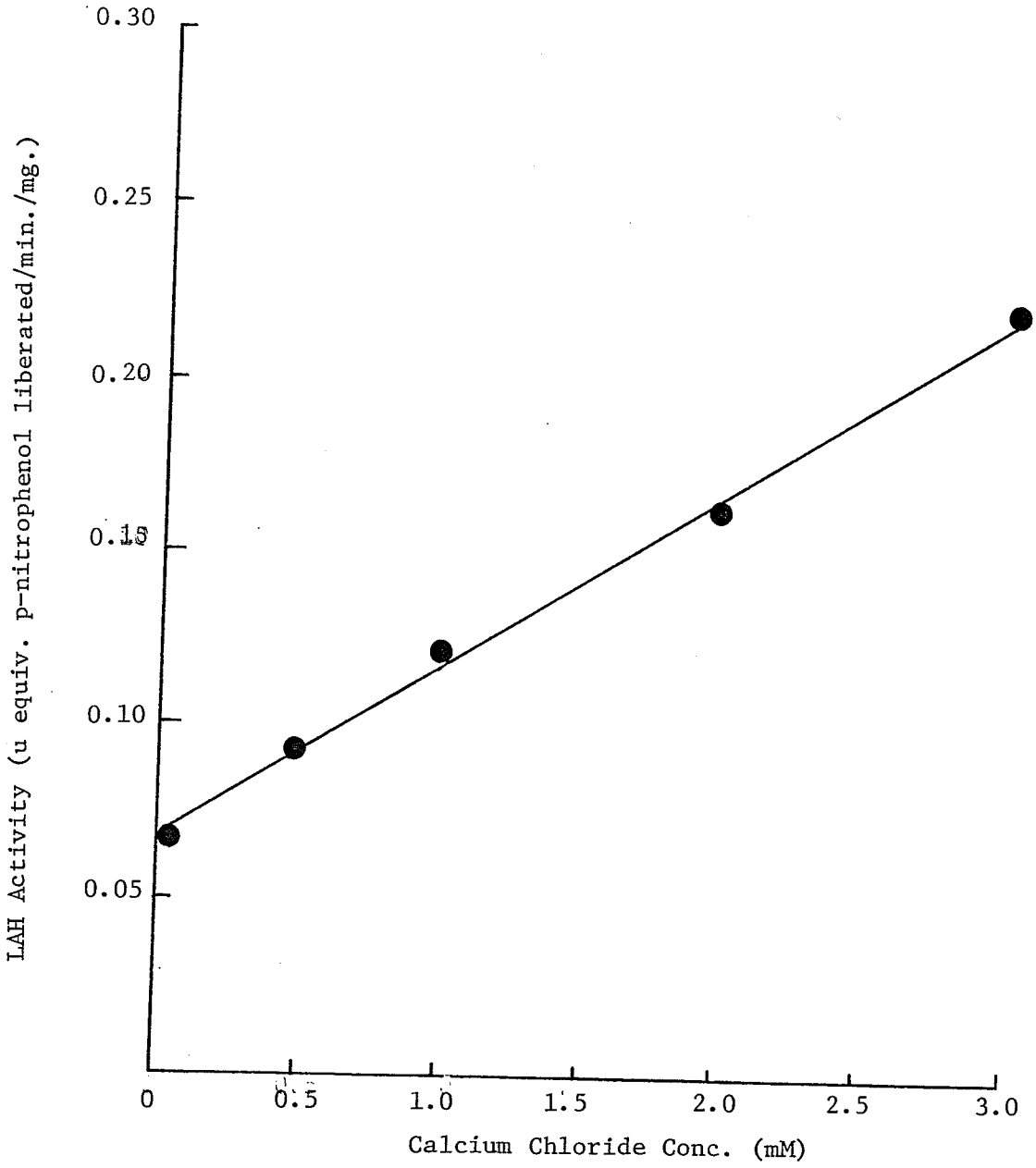


Figure 26. Effect of $CaCl_2$ on Fababean Lipolytic Acyl Hydrolase (p-nitrophenyl palmitate substrate).

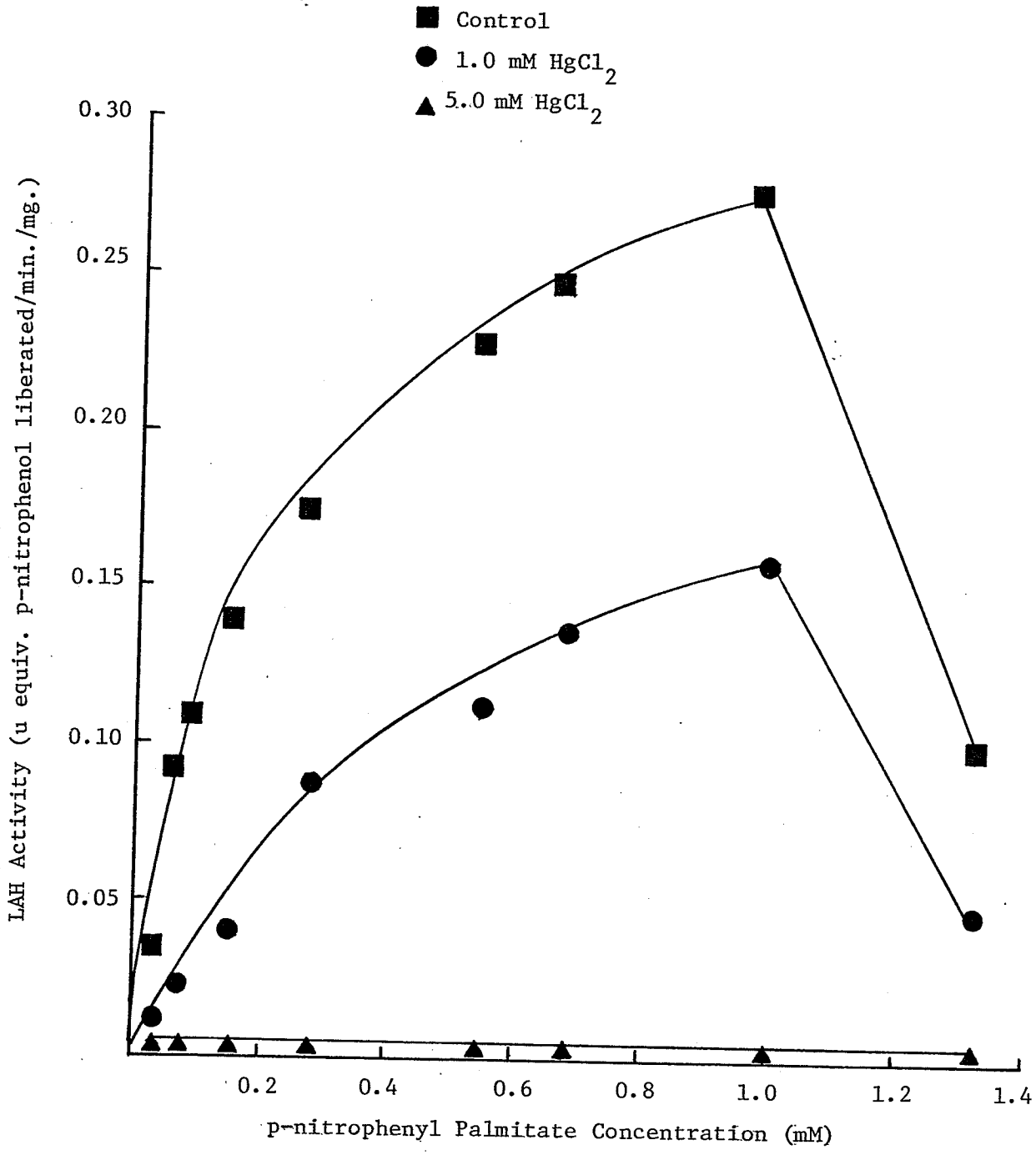


Figure 27. The Effect of $HgCl_2$ on Fababean Lipolytic Acyl Hydrolase (p-nitrophenyl palmitate substrate).

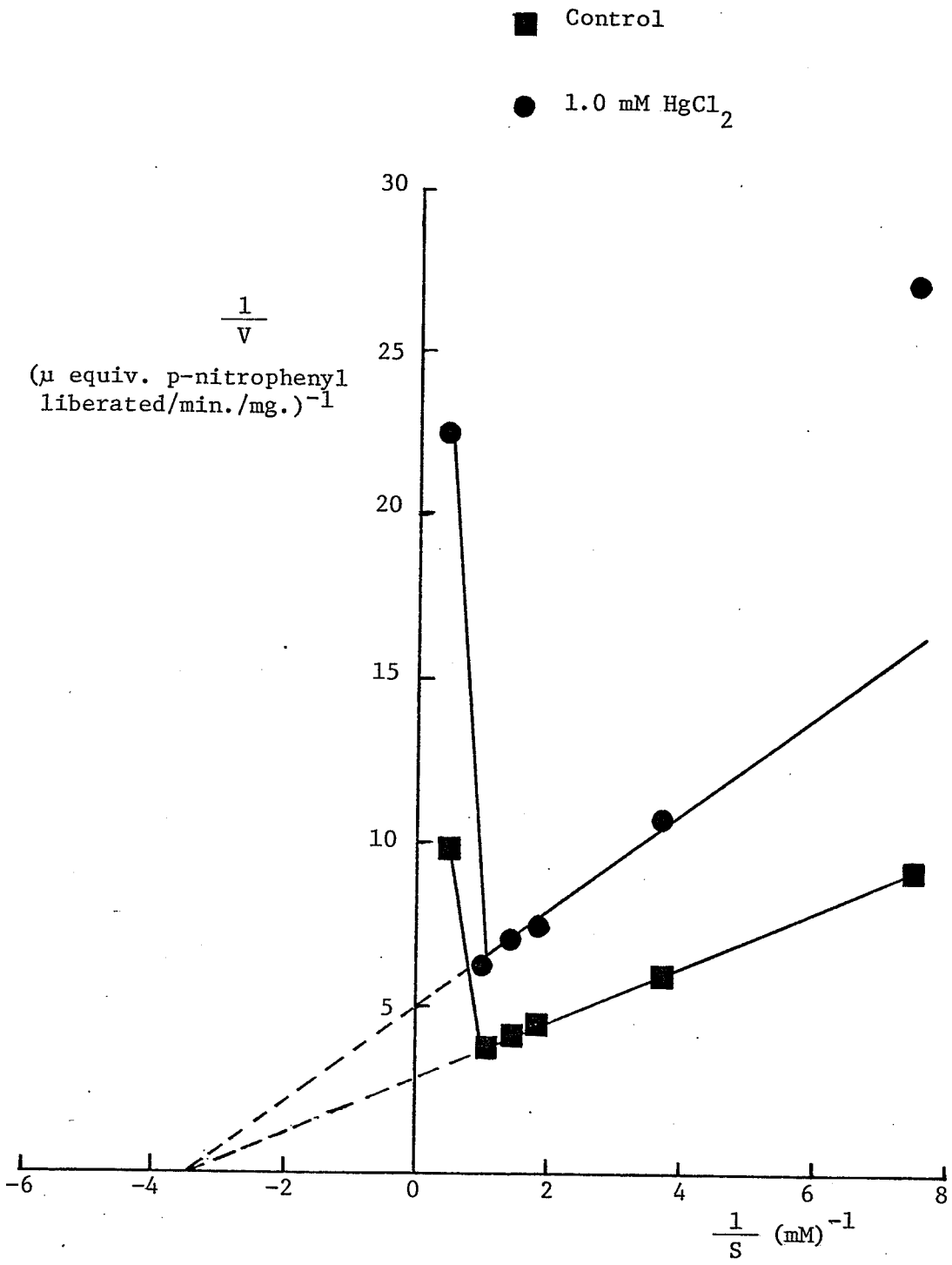


Figure 28. The Lineweaver-Burk Plot of the Effect of $HgCl_2$ on Fababean Lipolytic Acyl Hydrolase (p-nitrophenyl palmitate substrate).

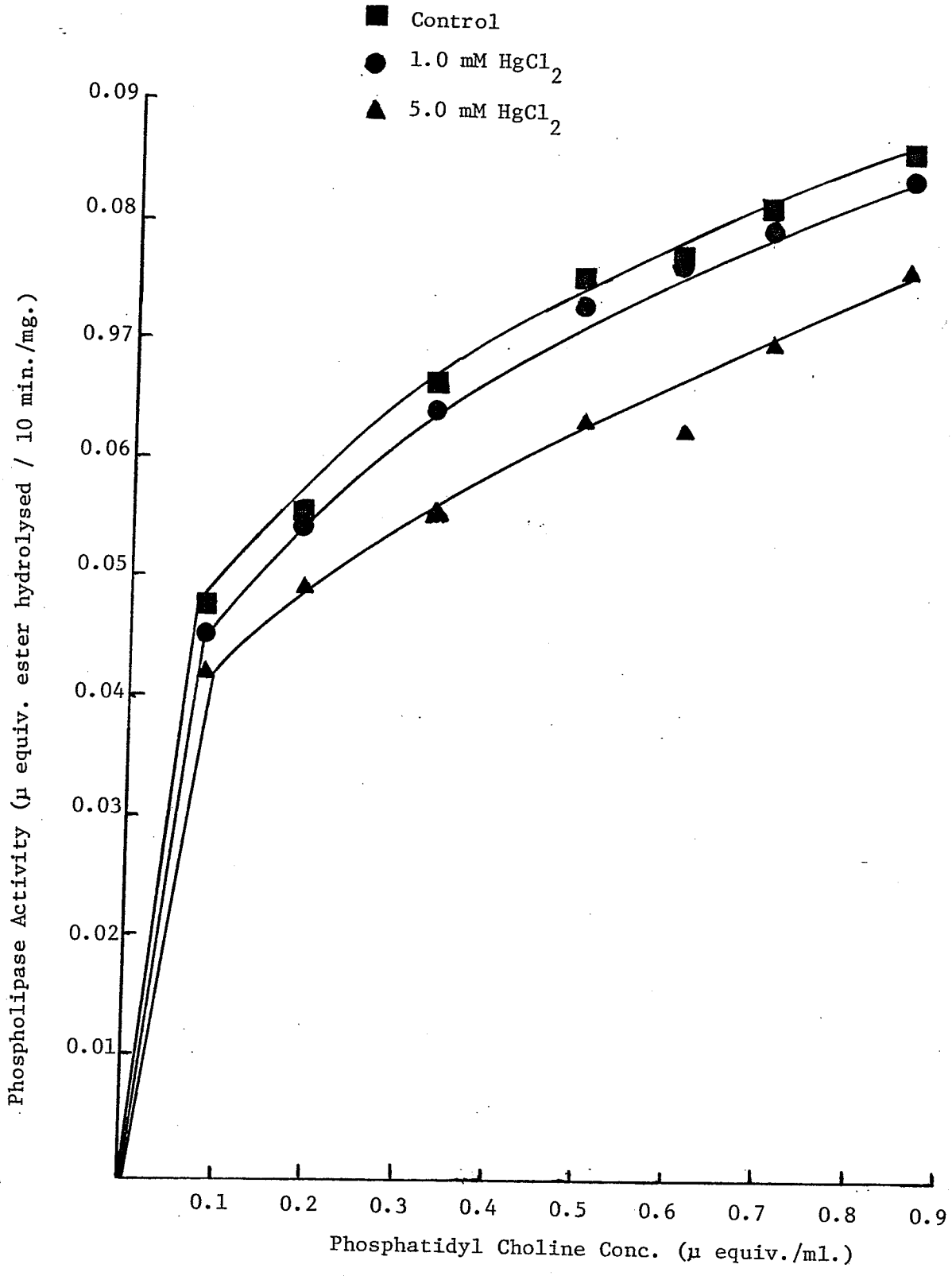


Figure 29. The Effect of $HgCl_2$ on Fababean Lipolytic Acyl Hydrolase (phosphatidylcholine substrate).

plot of the data from Figures 27 and 29. The Lineweaver-Burk plots (Figures 28 and 30 for p-nitrophenyl palmitate and phosphatidylcholine hydrolysis respectively) show that inhibition by mercuric ions is noncompetitive. Both the vertical intercept and slope of the reciprocal plots are affected and converge on the abscissa indicative of noncompetitive inhibition.

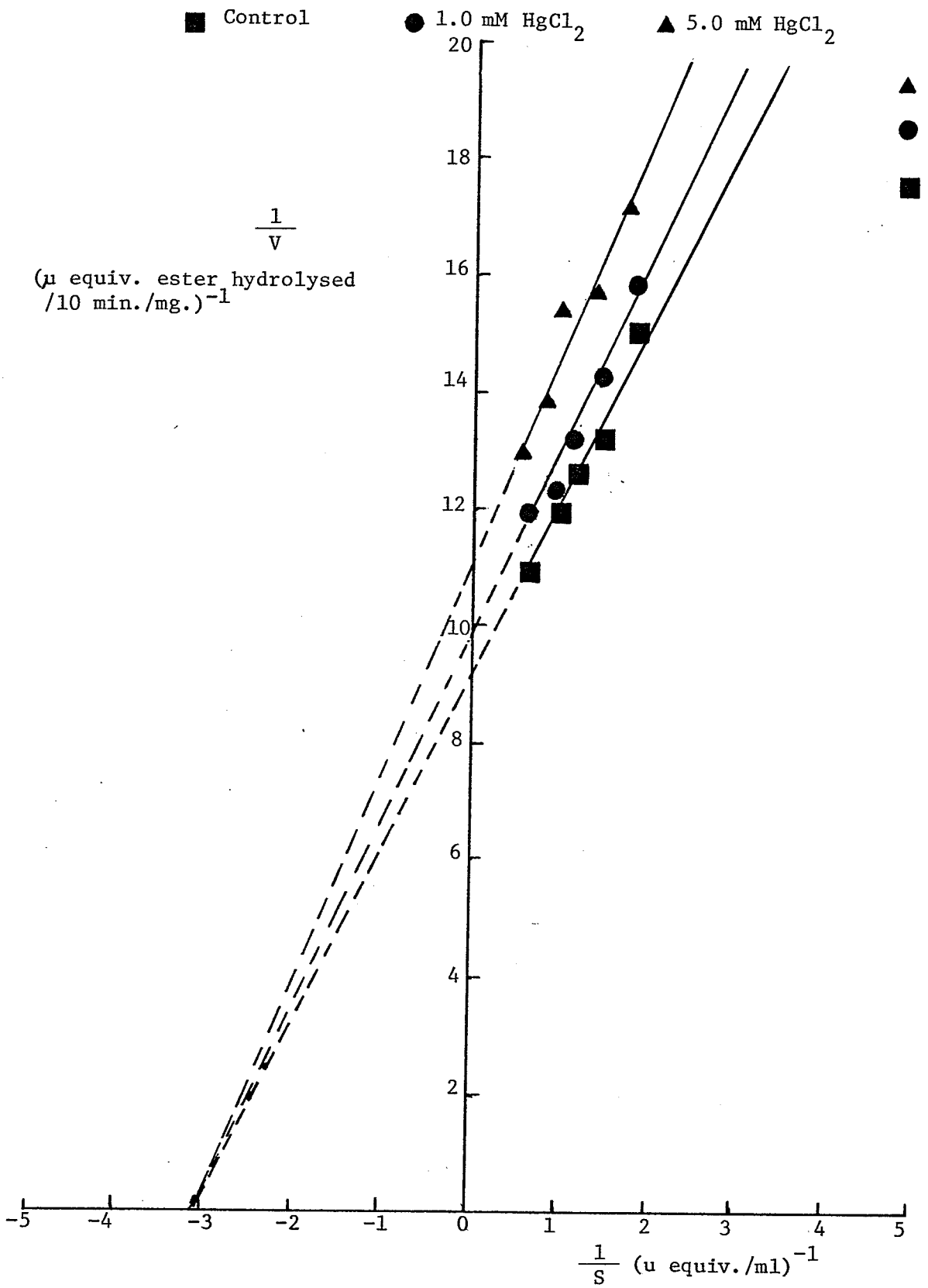


Figure 30. Lineweaver-Burk Plot of the Effect of $HgCl_2$ on Fababean Lipolytic Acyl Hydrolase (phosphatidylcholine substrate).

6. DISCUSSION

Crude preparations of fababean lipolytic acyl hydrolase were used to characterize the enzymic deacylation of a variety of lipid substrates. Although the use of crude extracts in kinetic studies is undesirable, it was necessitated by the fact that initial attempts at purification (acetone fractionation) lowered the specific activity of the fababean enzyme. This loss of activity was thought to be due to the separation of the lipid or lipoprotein fraction from the fababean protein extract, which was essential for maximum enzyme activity. A similar situation is encountered in the purification of plant and microbial lipases, which are present as a high molecular weight form termed "fast" lipase. This form of lipase is a multimolecular aggregate of enzyme molecules with strong associations with lipids, especially phospholipids (Brockerhoff and Jensen, 1974). This complex is easily denatured or inactivated by the application of techniques which disrupt the hydrophobic bonds of the enzyme aggregate. Galliard (1971a) reported difficulties in purification of potato tuber lipolytic acyl hydrolase due to a close association of enzyme activity with a major protein component. A possible solution to the problem of plant enzyme purification is the application of affinity chromatography, which has been used successfully in the purification of soybean lipoxygenase (Grossman et al., 1972; Sekiya et al., 1978).

The lipolytic acyl hydrolase-catalyzed hydrolysis of natural and synthetic esters is a function of the "concentration" of the emulsion of the substrate, the reaction occurring at the mixed

micelle-water interface. The Triton X-100-lipid mixed micelle system allows a direct kinetic investigation of the interaction of the enzyme and the lipid-water interface, because it provides a system in which the concentration of substrate in the interface can be varied and the activity can be followed by standard kinetic techniques (Deems et al., 1975). Although a wide variety of detergents or surfactants have been used for the emulsification of substrates, the use of Triton X-100 has been favored because it is relatively less destructive to proteins, has a defined chemical structure, and its use does not introduce charged species into the system, although some enzymes such as porcine pancreatic phospholipase require a negative surface charge on the micelle even though the substrate molecule itself may be an electrically-neutral lipid such as phosphatidylcholine (de Haas et al., 1970).

In lipolysis the enzyme has to seek out the substrate, as the latter is immobilized (except for lateral motion) in a larger matrix, this being the aggregated substrate molecules surrounded by water. The term "supersubstrate" (S_S) has been proposed (Brockerhoff, 1974) to describe the matrix in which a substrate is embedded. In lipolytic reactions this matrix may be the surface of a triglyceride droplet consisting of an aggregate of many substrate molecules, or it may be the surface of a micelle modified by the inclusion of nonsubstrates such as Triton X-100. Lipolysis is a one-substrate reaction, and is essentially zero-order at higher substrate concentrations. In those reactions that are subjected to substrate inhibition, however, the reaction is not zero-order, with the excess substrate acting as an inhibitor (Bergmeyer, 1978). At infinite substrate concentration, the velocity (v) becomes Vmax, the maximum

velocity of the reaction. Apparent values for Vmax can be determined by measuring the initial velocity (v) at several substrate concentrations, the data being plotted directly or in the form of a Lineweaver-Burk plot. The experimental data shown in Figures 3, 5, 7, 9, 11, 13, 16, and 18 are rectangular hyperbolas. Those representing the long-chain p-nitrophenyl esters (Figures 3, 5, 7 and 9) show marked deviations at higher substrate concentrations (substrate inhibition). The data for 1,2 dilinolein and phosphatidic acid (Figures 16 and 18) show atypical curves due to difficulties in emulsifying these substrates. The activity curve can be regarded as an adsorption isotherm in that it illustrates the progressive adsorption of the enzyme at an interface of increasing concentration. The rate of reaction is low when the concentration of the interface is small as few enzyme molecules are adsorbed. As the concentration of the interface increases so does the rate of reaction to a maximum (Vmax), the point at which all enzyme molecules are simultaneously adsorbed. The concentration, expressed as a molarity of the substrate, is not regarded as a weight, but as the amount of substrate in the emulsion (Brockerhoff and Jensen, 1974). The values of Vmax summarized in Table 1 should be considered as apparent values reflecting the conditions of assay under which they were determined.

Lipolytic reactions can formally be treated so that they follow Michaelis-Menten kinetics. However, the fact that reaction takes place at an oil-water interface causes some unique considerations to be given to the derivation of kinetic equations which include the role played by the interface in the reaction. In lipolysis the formation of the Michaelis complex is preceded by the adsorption of lipolytic acyl hydrolase to the micelle-water

interface which is the supersubstrate (S_s) in which the substrate is embedded (Brockerhoff, 1974). This equation can be visualized as:

$$E + S_s \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_s \qquad ES_s + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES_sS \qquad \underset{k_6}{\overset{k_5}{\rightleftharpoons}} ES_s + P$$

This equation can be shown to describe formally a one-substrate enzymic reaction with two intermediate complexes (Mahler and Cordes, 1971; Plowman, 1972), and can be resolved for $v = dP/dt$:

$$v = \frac{(k_1k_3k_5\ S - k_2k_4k_6\ P)\ e_o}{(k_2k_5) + (k_2k_4) + (k_3k_5) + k_1\ (k_3 + k_4 + k_5)\ S + k_6\ (k_2 + k_3 + k_4)\ P}$$

In the beginning of the steady state, $P = 0$, and

$$v = \frac{k_1k_3k_5\ Se_o}{k_2k_5 + k_2k_4 + k_3k_5 + k_1\ (k_3 + k_4 + k_5)\ S.}$$

This equation can be shown to be equivalent to the Michaelis-Menten equation by appropriate substitution:

$$\frac{k_3k_5\ e_o}{k_3 + k_4 + k_5} = V \quad \text{and,}$$

$$\frac{k_2k_5 + k_2k_4 + k_3k_5}{k_1\ (k_3 + k_4 + k_5)} = K_m, \text{ to give}$$

$$v = \frac{k_1 (k_3 + k_4 + k_5) VS}{Km\ k_1 (k_3 + k_4 + k_5) + Sk_1 (k_3 + k_4 + k_5)}$$

$$v = \frac{k_1 (k_3 + k_4 + k_5) VS}{k_1 (k_3 + k_4 + k_5) (Km + S)}$$

$$v = VS / Km + S$$

It can be demonstrated that the introduction of any number of unimolecular steps between different enzyme forms, all containing the same number of substrate or product molecules, does not alter the form of the rate equation. What does change is the composition of these parameters or coefficients in terms of individual rate constants (Mahler and Cordes, 1971). The Michaelis constant (Km) may be defined as the interface concentration for which the rate is V max/2,

Calculated values of Km obtained with fababean lipolytic acyl hydrolase are summarized in Table 1. Because the lipid substrates are not water-soluble and were present in micellar dispersions, the absolute Km values probably have little meaning, but the relative values give some information about the enzyme affinity for different substrates (Galliard, 1971a). Since the enzyme requires a substrate in a solubilized form, the Km values may also reflect the degree of solubility of each particular substrate. At pH 8.5, Km values for the hydrolysis of p-nitrophenyl esters range from 0.22 mM (p-nitrophenyl myristate) to 1.43 mM (p-nitrophenyl acetate). Activity with the p-nitrophenyl long-chain fatty acid esters was constant, with a slight rise in activity with decreasing fatty acid chain

length. However, activity with p-nitrophenyl acetate was several orders of magnitude greater, at pH 8.5 and 7.5. It is difficult to state whether this result demonstrates an enzyme specifity towards shorter chain fatty acids, or if the higher levels of activity demonstrate a higher degree of solubility with decreasing fatty acid chain length. It was observed that the shorter chain p-nitrophenyl esters (p-nitrophenyl acetate and laurate) solubilized much more readily, whereas p-nitrophenyl stearate (C_{18}) emulsions were very unstable and could not be assayed at pH 7.5 for this reason. Galliard (1971a) reported increasing activity with decreasing fatty acid chain length, when methyl-fatty acid esters were assayed with potato tuber acyl hydrolase. With p-nitrophenyl esters of fatty acids, no definite trends were reported; the C_{16} and C_8 acids showed similar rates of hydrolysis whereas the C_{12} ester was hydrolysed more slowly. The calculated Km for p-nitrophenyl palmitate (pH 8.5) is 0.29 mM which compares favorably with the 0.5 mM value reported for potato lipolytic acyl hydrolase hydrolysis of this same substrate (Galliard, 1971a). Other values reported by Galliard (1971a) include 0.7 mM (p-nitrophenyl stearate) and 2.0 mM (p-nitrophenyl acetate) which compare with 1.82 mM and 1.43 mM respectively for hydrolysis by the fababean enzyme.

In terms of phospholipid degradation a Km value of 0.18 mM (0.36 microequivalents of ester/ml) is reported for phosphatidylcholine at pH 5.6 (the optimum pH for fababean phospholipase activity). This compares with 1.7 mM reported for potato tuber enzyme (Hirayama et al., 1975), and with 0.54 mM reported for the deacylating enzyme from potato leaves (Matsuda and Hirayama, 1979). Hydrolysis of 1,2 dilinolein by the fababean enzyme gave an apparent Km of 1.43

microequivalents of ester/ml (0.72 mM). This should be regarded as an approximate value due to the relative insolubility of this substrate, and the difficulties encountered during assay. The same problems were apparent for the phosphatidic acid substrate, thereby not permitting a determination of Vmax and Km. The data obtained are reported here to give evidence to the proposition that fababean lipolytic acyl hydrolase may be active towards these two substrates. The comparatively low levels of activity are probably a reflection of the low degree of solubility obtained.

The tests with crude hog pancreatic lipase were performed with an aim towards determining the possible enzyme specificity of fababean acyl hydrolase. The lipase which is active towards the ester linkages of triacylglycerols demonstrated very low activity with phosphatidylcholine at pH 5.6 and 7.4 This would indicate that the deacylation of phospholipids by fababean acyl hydrolase may be attributed to a phospholipase activity which may be distinct from fababean lipase.

Assays with DL-α-glycerophosphate and β-glycerophosphate were performed to test the specificity of the method used to measure fababean phospholipase activity (Snyder and Stephens, 1959; Renkonen, 1961). Both DL-α-glycerophosphate and β-glycerophosphate contain only phosphoric ester linkages and should give a negative result (no color development) when the acyl ester determination was performed. A negative result was obtained with both substrates indicating that the decrease in ester content reported here as indicative of fababean phospholipase activity may be attributed to a deacylation process at positions C-1 and/or C-2 of the phospholipid substrates. This would merit a definition of phospholipase A- and B-like

activities in fababeans.

The optimum pH for fababean p-nitrophenyl ester hydrolase activity is 8.5, while an optimum of 5.6 was determined for phospholipase activity. These values compare favorably to those of other p-nitrophenyl ester-hydrolyzing enzymes (Galliard, 1971a; Hasson and Laties, 1976a), and to other phospholipases (Galliard, 1970; Shepard and Pitt, 1976b; Galliard et al., 1976; Matsuda and Hirayama, 1979). The pH optimum not only depends on the nature and ionic strength of the buffer, but also generally varies with both temperature and substrate concentration, since the Km of most enzymes changes with pH. pH profiles are most meaningful if the enzyme is kept saturated with substrate at all pH values tested (Lehninger, 1976; Bergmeyer, 1978). In choosing the buffer, care must be taken that the pK of the buffer is as close as possible to the pH optimum of the reaction. For this reason a diethanolamine-HCl buffer was preferred over the more common Tris-HCl buffer.

The optimum temperature of fababean lipolytic acyl hydrolase was found to be 37°C, this figure corresponding well to the optima reported in the literature for other acyl hydrolases (Hirayama et al., 1975; Hasson and Laties, 1976b; Franson et al., 1978; Matsuda and Hirayama, 1979). Assays could not be performed at temperatures above 37°C due to nonenzymic hydrolysis of the substrate, however, temperature stability data support this figure (37°C) as fababean lipolytic acyl hydrolase is rapidly denatured at temperatures slightly above this. The fababean enzyme was very susceptible to heat inactivation. Ten minutes' incubation at 47°C resulted in nearly 50% of original enzyme activity to be inhibited, while exposure to 75°C for two minutes completely inhibited enzyme activity.

Similar temperature stability data were reported by Galliard (1971a), whose potato tuber acyl hydrolase lost 50% of its activity after incubation at 60°C for three minutes.

Variations in the level of Triton X-100 used to emulsify p-nitrophenyl palmitate had a pronounced effect on the activity of fababean lipolytic acyl hydrolase. A concentration of 6 mg. Triton/ml. was found to be optimal, and concentrations higher than this were found to be inhibitory, presumably due to a dilution effect on the available substrate at the interface (Dennis, 1973b). Concentrations lower than 6.0 mg./ml. did not result in optimal enzyme activity, as enough Triton was not present to enable all the substrate to form the desired mixed micelles. Figure 25 demonstrates the effect of Triton concentration used to solubilize phosphatidylcholine. An optimum of 4.0 mg./ml. Triton is indicated in a broader range of peak activity. Higher levels of Triton were found to be inhibitory, again probably due to dilution of the phosphatidylcholine at the water-micelle interface. Similar data were reported by Dennis (1973b) with phospholipase A_2 from Naja naja venom. A Triton concentration of 4.0 mg./ml. corresponds to a molar ratio of 2:1 Triton/phospholipid.

Studies with calcium chloride, a potential activator, indicated that p-nitrophenyl ester hydrolase activity is stimulated up to a concentration of 3.0 mM, although the Ca^{2+} ion was not essential for activity. The activity remained constant, however when phosphatidylcholine was assayed with increasing concentrations of calcium chloride, indicating that the Ca^{2+} ion had no observable effect on the fababean phospholipase. Calcium ions remove unionized fatty acids from the interface through the formation of insoluble calcium

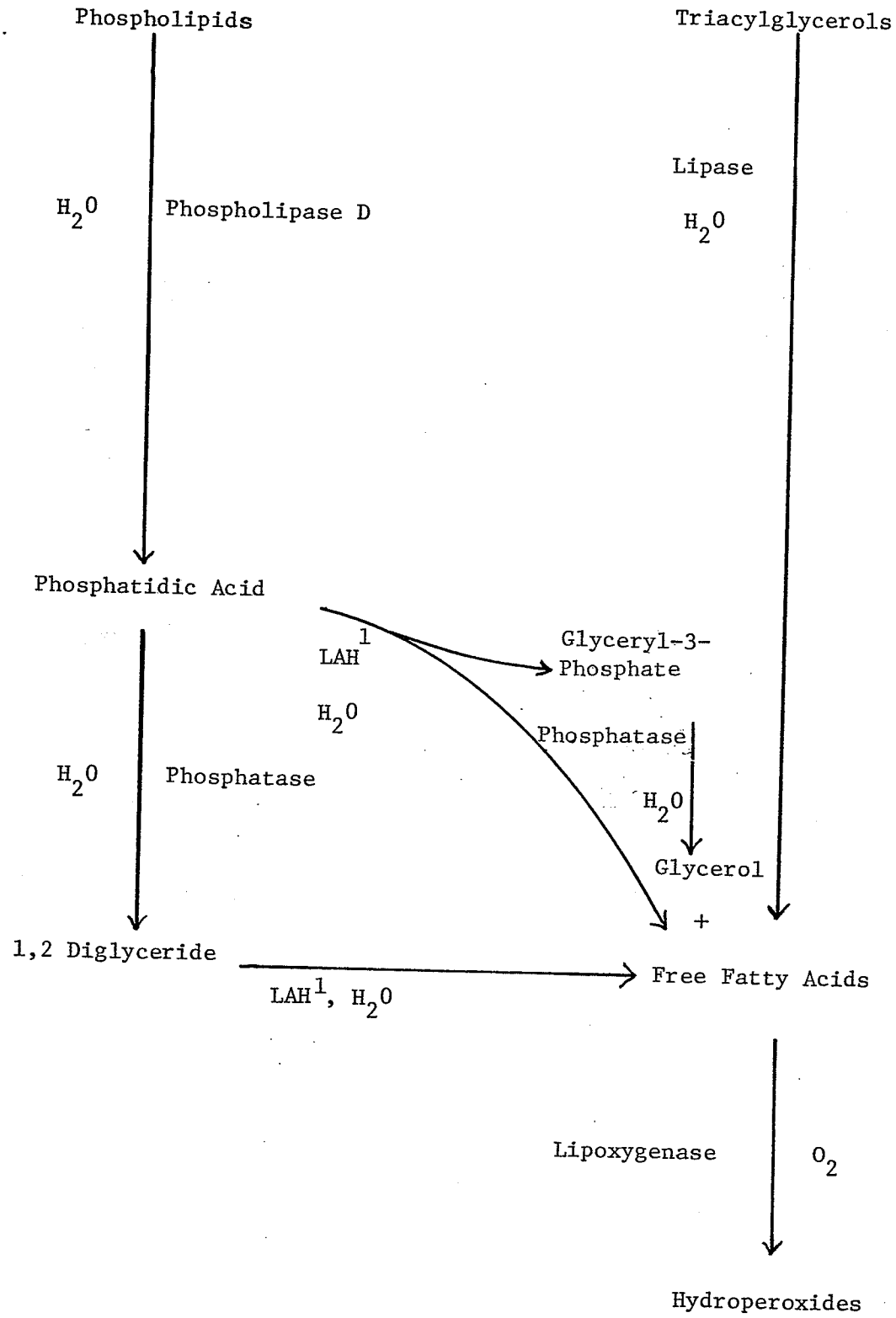
soaps, thereby allowing the enzyme greater mobility at the interface and a corresponding increase in the reaction rate. The phospholipase assay was not stimulated by additional calcium ions, but this may be explained by the fact that sufficient calcium ions were already present in the crude fababean extract and the effect of the additional salts would be negated. This does not necessarily mean that fababean phospholipase is not activated by calcium chloride. The rise in p-nitrophenyl ester hydrolase activity with addition of calcium chloride may be explained by the fact that this activity is several orders of magnitude greater than the phospholipase activity (both considered without additional calcium ion). Therefore a much greater concentration of freed fatty acids would be present at the interface and additional calcium chloride would be necessary for optimal rates of enzyme activity.

Studies with the heavy metal salt mercuric chloride resulted in inhibition of fababean lipolytic acyl hydrolase activity, although the degree of inhibition varied with the substrate assayed. The Hg^{2+} ion strongly inhibited (40% loss of activity) hydrolysis of p-nitrophenyl palmitate at a concentration of 1.0 mM, while 5.0 mM $HgCl_2$ completely inhibited enzyme activity. Quite different results were obtained, however, with phosphatidylcholine as substrate. Inhibition of activity was slight even at a concentration of 5.0 mM $HgCl_2$, where only 6% of original activity was lost. It was shown from the double reciprocal plot of the $HgCl_2$ inhibition studies that the inhibition was linear and noncompetitive. A noncompetitive inhibitor can combine with either the free enzyme or the enzyme-substrate complex, binding to a site on the enzyme other than the active site, often to deform the enzyme. This deformation causes a reduction in the rate of enzyme-substrate complex formation, and once formed,

this complex does not decompose at the normal rate to yield products. These effects are not reversed by increasing the substrate concentration (Lehninger, 1976).

The overall objective of this study was the determination of release of free fatty acids from the phospholipid fraction of fababean lipids by endogenous lipolytic acyl hydrolase. Free fatty acids, especially linoleic acid, are the substrates for lipoxygenase which has been identified in fababean (Eskin and Henderson, 1974a,b). Conjugated cis, trans hydroperoxides are the products of lipoxygenase catalysis, and these in turn are presumably further degraded to highly volatile short-chain aldehydes and ketones, characteristic of rancidity. Various other approaches have been taken to determine the mechanism of fababean lipid breakdown. These include the study of a lipase (Dundas et al., 1978), phospholipase D (Atwal et al., 1979) and phosphatase (Atwal and Eskin, unpublished data). A summary of these data (Henderson et al., 1979) has led to the proposition of a sequential hydrolytic and oxidative enzymic pathway, by which fababean endogenous lipids may be degraded upon tissue disruption (Figure 31). This scheme is based on the one proposed by Henderson et al. (1979) and has been modified to incorporate recent data. The hydrolytic stage results in the breakdown of the major fababean lipids (phospholipids and triacylglycerols) to release free fatty acids, for which fababean lipoxygenase is highly specific (Eskin and Henderson, 1974a). Dundas et al. (1978) reported a pH optimum of 8.5 for fababean lipase which is the same value reported here for the hydrolysis of p-nitrophenyl esters. The pH optimum of fababean phospholipase D is 5.7 (Atwal et al., 1979), which is close to the value of 5.6 reported here as the optimum pH for the deacylation of phosphatidylcholine.

Figure 31. Proposed Pathway for the Enzymic Degradation of Endogenous Lipids Upon Disruption of Vicia faba minor tissues.



[1] LAH = Lipolytic Acyl Hydrolase

Shepard and Pitt (1976b) separated two lipolytic activities from potato tuber, a phospholipase (pI = 4.24 $\pm$ 0.35) and an acyl hydrolase active with p-nitrophenyl palmitate (pI = 7.29 $\pm$ 1.40). The data concerning fababean lipolytic enzymes suggest that a similar enzyme activity hydrolyses triacylglycerols and the 'artificial' p-nitrophenyl esters, and that a separate acyl hydrolase activity is responsible for phospholipid degradation (Henderson *et al.*, 1979).

In terms of the results reported in this thesis, there is room for additional research. The outstanding problem concerns the purification of fababean lipolytic acyl hydrolase, with an aim towards determining the specificities of the enzyme activities. Many of the problems encountered during the course of this research could be attributed to the fact that a crude enzyme preparation was used in all the kinetic studies. The results obtained in the Triton X-100, $HgCl_2$ and pH studies suggest that two separate enzyme activities may be present in the fababean extract. Galliard and Dennis (1974b) separated several isoenzymic activities that were originally thought to be due to a single enzyme in a crude extract (Galliard 1971a). The most promising purification procedures are those of Hirayama *et al.* (1975), Hasson and Laties (1976), and Shepard and Pitt (1976). The application of affinity chromatography might also be warranted. A second area for additional investigation might be the optimization of the assay conditions. The values reported for Km and Vmax are reflective of the assay conditions used to obtain them. More comparable values would be obtained if calcium chloride was used as an activator in the standard procedure, if the level of Triton X-100 used to stabilize the p-nitrophenyl esters was increased to 6.0 mg./ml. and if the temperature of assay was increased to 37°C.

A final area of concern is in regard to the choice of emulsifying agent. Triton X-100 has had wide-spread application in the study of lipolytic enzymes, and its use has many theoretical and practical advantages. However, the difficulties encountered in solubilizing 1,2 dilinolein, phosphatidic acid and certain of the p-nitrophenyl esters, suggest that the use of other dispersion agents, such as the bile salts, might be warranted. The use of bile salts might be beneficial due to the fact that they impart a negative surface charge to the mixed micelle, which is essential to certain phospholipases (Brockerhoff, 1974).

In terms of the mechanism outlined in Figure 31, further characterization of the enzyme activities is warranted. A further area of investigation would be the mechanism of short-chain aldehyde and ketone production in fababean from the hydroperoxide products of lipoxygenase catabolism. Two types of enzyme systems are possible, a hydroperoxide isomerase as isolated from flaxseed (Zimmerman and Vick, 1970), or a hydroperoxide cleaving enzyme as studied in cucumber (Galliard and Phillips, 1976; Phillips and Galliard, 1978) and in potato plant leaves (Matthew and Galliard, 1978).

The purpose of this thesis, the detection and characterization of lipolytic acyl hydrolase activities in fababean has been successfully attained. The properties which have been determined have been shown to conform generally with plant, animal and microbial deacylating enzymes so far investigated. This study has been conducted with an aim towards the control of enzymic lipid degradation in stored processed fababeans. The heat-stability data presented here and in the other reports concerning fababean lipid-degrading enzymes suggest that a heat-treatment step might be the most appropriate method to control

the development of rancidity. The use of chemical inhibitors would be of questionable value, as this study demonstrated that 5.0 mM $HgCl_2$ had little effect on the deacylation of phosphatidylcholine. In addition, mercuric chloride could not be used in material intended for human or animal consumption.

7. SUMMARY

1. The deacylation of various lipid substrates was determined in fababean (Vicia faba L var. minor). These enzymic activities can be described with the general name "Lipolytic Acyl Hydrolase".
2. Due to the properties of the crude extracts of fababean lipolytic acyl hydrolase, acetone precipitation was unsatisfactory for the concentrating and/or purification of the enzyme. Crude extracts were used for the kinetic studies.
3. A linear reaction with time for enzyme concentration against the rate of hydrolysis was obtained for extracts of fababean lipolytic acyl hydrolase.
4. Hydrolysis of p-nitrophenyl esters was performed at pH 7.5 and 8.5, with higher activities observed at pH 8.5. Levels of activity were higher with shorter-chain fatty acid derivatives, although this may be a reflection of the degree of solubility of each substrate rather than an enzyme preference for shorter-chain fatty acids.
5. Theoretical maximum rates of hydrolysis could not be reached for the longer-chain p-nitrophenyl esters, because the high levels of substrate needed for Vmax to be reached, caused substrate inhibition.
6. Hydrolysis of phospholipid substrates was carried out at pH 5.6 and 7.5 with greater activity demonstrated at pH 5.6
7. Difficulty was obtained in obtaining suitable emulsions with some of the phospholipid substrates, suggesting that other methods of dispersing the lipid might be employed.
8. Hydrolysis of phospholipid substrates was not, in most cases, subject to substrate inhibition.

9. Assays with crude hog pancreatic lipase resulted in very low levels of activity suggesting that phospholipase activity may be distinct from a lipase fraction of the crude fababean extract.
10. Experiments to test the specificity of the method used to determine phospholipase activity, indicate that the fababean enzyme may warrant a designation as a phospholipase A or B.
11. Calculated values of Km correspond well to values reported for deacylating enzymes isolated from other plant, animal and microbial sources.
12. Lipolytic acyl hydrolase activity was demonstrated over a pH range of 6.0 - 9.0 with the optimum being 8.5. Phospholipase activity was demonstrated over a pH range of 4.5 to 8.5. The pH optimum was 5.6 with a possible secondary optimum of pH 7.5
13. The optimum temperature for enzyme activity was 37°C. In solution, the fababean lipolytic acyl hydrolase lost all lipolytic activity after two minutes exposure to 75°C.
14. An optimum concentration of 6.0 mg. Triton/ml. was determined for the stabilization of p-nitrophenyl substrates, while phospholipid substrates are stabilized with an optimum Triton concentration of 4 mg./ml.
15. Calcium chloride, in concentrations up to 3.0 mM, stimulated the hydrolysis of p-nitrophenyl palmitate, but had no effect on the rate of phospholipid hydrolysis.
16. Mercuric chloride completely inhibited p-nitrophenyl palmitate hydrolysis at a concentration of 5 mM; this concentration, however only inhibited 6% of activity when phosphatidylcholine was assayed. Inhibition was noncompetitive.

17. A sequential hydrolytic and oxidative enzyme pathway is proposed, which would account for the degradation of fababean endogenous lipids upon tissue disruption.
18. It is recommended that future work with fababean lipolytic acyl hydrolase be concerned with enzyme purification.
19. A heat-treatment stage is recommended as the most likely method of controlling rancidity development in stored processed fababean.

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